

RP 1623

National Science Foundation

Grant No NSF/RG/2005/HS/06

Final Report

**Strain-specific immunity to *Plasmodium vivax* asexual
erythrocytic stage antigens in Sri Lanka**

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Section 1

Information regarding Project/Project Personnel:

- i) Contract Number – NSF/RG/2005/HS/06
- ii) Title of the Project – Strain-specific immunity to *Plasmodium vivax* asexual erythrocytic stage antigens in Sri Lanka
- iii) Principal Investigator – Prof. Preethi V. Udagama-Randeniya
- iv) Co-Investigator – Dr Anusha Munasinghe-Gunasekera
Collaborator - Dr Shiroma Handunnetti
- v) Institute(s) where research was being carried out – Department of Zoology, University of Colombo
- vi) Date of award – 01.06.2005
- vii) Date of completion of Project – 30.11.2008 (NSF imposed deadline)
- viii) Total allocation of funds (Rs) – 2,016,500.00
- ix) Total spent (Rs) – 2,016,500.00
- x) Number of Research Students employed – 01 (Sept 2005 to Dec 2008)
01 (Oct 2007- Dec 2008)
- xi) Post graduate degree completed with dates – Two PhD thesis (one submitted in June, 2010 and the other to be submitted in August, 2010)
- xii) Number of Technical Assistants and/or labourers employed and period of service – None

- xiii) Publications/Communications arising from the project during the reporting period –

3. International peer reviewed publication

- (i) Thilan Wickramarachchi*, Prasad H Premaratne*, Sajani Dias*, Shiroma M Handunnetti, Anusha M Gunasekara, Dyann F Wirth and Preethi V Udagama-Randeniya (2010). **Evaluation of the level of genetic complexity of *Plasmodium vivax* infections using PvMSP3 α gene, in Sri Lanka.** Annals of Tropical Medicine and Parasitology, 104(2); 95-108.

(* Equal contribution to this work)

2. Manuscripts submitted to international peer reviewed journal -

- (i) Sajani Dias, Shirley Longacre, Ananias A Escalante and Preethi V Udagama-randeniya. **Genetic Diversity and Recombination at the C-terminal Merozoite Surface Protein-1 (*Pvmsp-1*₄₂) gene of *Plasmodium vivax* in Sri Lanka.** Manuscript submitted to the journal of Infection, Genetics and Evolution was reviewed and suggested editing of the manuscript is under way for resubmission (MEEGID/D/10/00167).
- (ii) Prasad H. Premaratne, Ananias A. Escalante, Preethi V. Udagama-Randeniya. **Genetic diversity of *Plasmodium vivax* Duffy Binding Protein under low transmission and unstable malaria in Sri Lanka.** Manuscript submitted to the *Gene*.

3. Finalised manuscripts to be submitted to international peer reviewed journals -

- (i) Sajani Dias, Maheshika Somarathna, Aresha Manamperi, Anusha M Gunasekera and Preethi V Udagama-Randeniya. **Evaluation of the genetic diversity of Domain II of *Plasmodium vivax* Apical Membrane Antigen 1 and the ensuing strain specific immune responses in patients from Sri Lanka.** Manuscript in preparation to be submitted to the *journal of Vaccine*.

4. Research communications -

Local:

- i) W T A Wickramarachchi, P H Premaratne, K V G S Dias, A D M Gunasekara, D F Wirth, S M Handunnetti and P V Udagama-Randeniya. **Genetic complexity of *Plasmodium vivax* infections in Sri Lanka.** 62nd Annual sessions of Sri Lanka Association of Advancement of Science, 2006. Page 3.
- ii) P H Premaratne, W T A Wickramarachchi, K L R Perera, S Bandara, C Chitnis, S Yazdani, S M Handunnetti and P V Udagama-Randeniya. **IgM antibody response to *Plasmodium vivax* Duffy Binding Protein in Sri Lanka.** 3rd Biennial Sessions of the Allergy and Immunology Society of Sri Lanka, 2006. Page 27.
- iii) P H Premaratne, K V G S Dias, W T A Wickramarachchi, K L R L Perera, S Bandara, C Chitnis, S Yazdani, S M Handunnetti and P V Udagama-Randeniya (2006). **Specificity of the antibody response to conformational and linear epitopes of *Plasmodium vivax* Duffy Binding Protein in Sri Lanka.** 3rd Biennial Sessions of the Allergy and Immunology Society of Sri Lanka, 2006. Page 25.
- iv) Prasad H Premaratne, K V G Sajani Dias, Shiroma M Handunnetti, Shams Yazdani, Chetan Chitins and Preethi V Udagama-Randeniya. **Patterns of anti-PvRII IgG and IgG isotypes to *Plasmodium vivax* Duffy Binding Protein in Sri Lanka.** 63rd Annual sessions of Sri Lanka Association of Advancement of Science, 2007. Page 11
- v) S. Dias, A. M. Gunasekera and P.V. Randeniya. **Sequence analysis of the apical membrane antigen 1 (AMA-1) of *Plasmodium vivax* in Sri Lankan isolates.** Proceedings of the 28th Annual sessions of the Institute of Biology, 2008. Page 42.
- vi) Sajani Dias, Thilan Wickramarachchi, Anusha Munasinghe-Gunasekera and Preethi Udagama- Randeniya. **Molecular Ecology of *vivax* malaria: genetic diversity of a**

malaria vaccine candidate antigen, Apical Membrane Antigen -1, in Sri Lanka.
National Annual Scientific Sessions 2008 on Ecological Medicine.

- vii) Prasad H Premaratne, Aresha Manamperi, Preethi V Udagama-Randeniya. **Analysis of genetic polymorphism of *Plasmodium vivax* Duffy Binding Protein ligand domain of Sri Lankan isolates.** 63rd Annual sessions of Sri Lanka Association of Advancement of Science, 2008. Page 11
- viii) Sajani Dias, Maheshika Somarathana, Aresha Manamperi, and Preethi Udagama Randeniya. **Reactivity of synthetic peptides of the *Plasmodium vivax* Apical Membrane Antigen-1 (PvAMA-1) domain II with natural antibodies in sera of *P. vivax* patients from Sri Lanka.** 4th Biennial Scientific Sessions of the Allergy and Immunology Society of Sri Lanka, July 2009. Page 24.
- ix) Prasad H Premaratne, A Rushdi Shakri, Chetan E Chitnis and Preethi V Udagama-Randeniya. **Characterization of anti-PvDBPII binding inhibitory antibodies of *Plasmodium vivax* malaria patients from Sri Lanka.** 4th Biennial Sessions of the Allergy and Immunology Society of Sri Lanka, July 2009. Page 18.
- x) S Dias and P V Udagama-Randeniya. **The Genetic Diversity of *P. vivax* Merozoite Surface Protein-1p42 (PvMSP-1p42) in Sri Lanka.** Proceedings of the 29th Annual Sessions of the Institute of Biology, Sri Lanka, September, 2009. Page 23.
- xi) Sajani Dias and Preethi Udagama-Randeniya. **Genetic diversity in the *Plasmodium vivax* C-terminal 42 kDa region of merozoite surface protein-1(PvMSP-1p42) in Sri Lanka.** 65th Annual sessions of Sri Lanka Association of Advancement of Science, December 2009. Page 6.

- xii) P.H. Premaratne, B.R. Aravinda, A.A. Escalante, P.V. Udagama-Randeniya. Phylogenetic relationship of *Plasmodium vivax* duffy binding protein gene between Sri Lanka and world wide isolates. Post-graduate sessions, University of Colombo, May 2010. Page 112

- xiii) Sajani Dias, Shirley Longacre, Preethi V. Udagama-Randeniya. **Interallelic and Intragenic Recombination at the Hypervariable Region of Merozoite Surface Protein-1 (MSP-1₃₃) of *Plasmodium vivax* in Sri Lanka.** Post-graduate sessions, University of Colombo, May 2010. Page 111-112.

- xiv) Sajani Dias, Thilan Wickramarachchi, Preethi V Udagama-Randeniya. **Comparison of antibody responses to asexual stage vaccine candidate antigens of *Plasmodium vivax* merozoite surface protein-1 and apical membrane antigen-1 in Sri Lanka.** 5th Biennial Sessions of the Allergy and Immunology Society of Sri Lanka, July 2010. Page 14. (Won the 1st prize for scientific papers in Basic Immunology).

- xv) Prasad H Premaratne, Preethi Udagama-Randeniya. **Strain-specific and strain-transcending antibody responses directed against *Plasmodium vivax* duffy binding protein region II in Sri Lanka.** 5th Biennial Sessions of the Allergy and Immunology Society of Sri Lanka, July 2010. Page 15. (Won the 2nd prize for scientific papers in Basic Immunology).

International:

- i) Prasad H Premaratne, K V G Sajani Dias, Shiroma M Handunnetti, Shams Yazdani, Chetan Chitins and Preethi V Udagama-Randeniya. **Immunoepidemiology of PvRII, a putative vaccine candidate representing *Plasmodium vivax* Duffy Binding Protein, in Sri Lanka.** 56th of the Annual Meeting of the American Society of Tropical Medicine and Hygiene, Philadelphia, USA, Supplement to the American Journal of Tropical Medicine and Hygiene 77 (5). Page 55-56

- ii) Dias S, Munasinghe-Gunasekera A, and Udagama-Randeniya P. **Genetic Diversity at the Domain II Locus of *Plasmodium vivax* Apical Membrane Antigen-1 (PvAMA-1) in Sri Lankan Isolates.** The Annual Meeting of the Royal Society of Tropical Medicine and Hygiene, September 2008, Brighton, UK. Page 35.
- iii) Prasad H Premaratne, Sajani Dias, Aresha Manamperi, Shiroma M Handunetti, Preethi V Udagama-Randeniya. **Genetic diversity of the critical binding motif of *P. vivax* Duffy Binding Protein in Sri Lanka.** 57th of the Annual Meeting of the American Society of Tropical Medicine and Hygiene, New Orleans, USA. 2008.
- iv) Sajani Dias, Thilan Wickramarachchi, Shirley Longacre and Preethi V. Udagama-Randeniya. **Strain specific immune responses against *Plasmodium vivax* Merozoite Surface Protein-1p42 (PvMSP-1p42) and p19 (PvMSP-1p19) strains in Sri Lanka.** Accepted for presentation at the 14th International Congress of Immunology, August 2010, Kobe, Japan.

Section 2

Executive Summary of the Project:

Characterization of genetic diversity among locally prevalent parasite strains and the ensuing strain-specific immunity will be particularly important in a specific geographical setting for development of vaccine constructs and for planning future vaccination strategies.

The present study characterized the prevailing genetic diversity of three major asexual stage putative vaccine antigens of *Plasmodium vivax*, 42 kDa fragment of the Merozoite Surface Protein -1 (MSP-1₄₂), Apical Membrane Antigen-1 domain II (AMA-1DII) and the Duffy Binding Protein region II (PvDBPII), in patients infected with *P. vivax* malaria from two endemic areas, Anuradhapura and Kataragama, in Sri Lanka, where unstable malaria prevails with low transmission, and from a malaria non-endemic area, Colombo, where the patients contracted the infections while visiting areas endemic to vivax malaria. Based on nucleotide sequences, extensive allelic polymorphism was evident in the N-terminal region of MSP-1₄₂ (*Pvmsp-1₃₃*), *Pvama-1dII* and *PvdbpII*, due to recombination, mutations, natural selection and genetic differentiation. Nucleotide sequences of *Pvmsp-1₄₂* (N=95), *Pvama-1dII* (64) and *PvdbpII* (100), generated 27, 21 and 33 amino acid (a.a) haplotypes, respectively. Clinical isolates with amino acid haplotypes available for all three antigens (N=24) revealed uniquely different haplotype combinations in each isolate. Conversely, PvMSP-1₁₉, domain II loop of PvAMA-1 and the six a.a. in the critical binding domain of PvDBPII responsible for direct binding with the Duffy antigen on human RBC, showed 100% conservation of the amino acid sequences.

In order to evaluate whether sequence differences among each antigen was indicative of strain-specific immunity, amino acid sequences of PvMSP-1₄₂, PvAMA-1DII and PvDBPII were aligned with the homologous total (IgM + IgG) antibody responses assayed by ELISA against the recombinant protein of each antigen, and the seven (P01-P07) PvAMA-1DII peptides. Though Anti-PvMSP-1₁₉ and anti-P04 antibody prevalence precludes strain-specific immune responses against the highly conserved PvMSP-1₁₉ and the P04 peptide on domain II loop of PvAMA-1, a protective immune response was clearly elicited to these two regions by the marked isotype switch from IgM to IgG with increasing exposure in areas of endemicity. Further, the presence of strain transcending (cross reactive) PvDBPII specific functionally active binding inhibitory antibodies were demonstrated among locally exposed *P. vivax* patients.

Thus this study may support the inclusion of PvMSP-1₁₉, P04 region of PvAMA-1 domain II and region II of PvDBP as veritable vaccine components for a multi component “cocktail” asexual stage vaccine against *P. vivax* malaria in Sri Lanka.

Section 3

Introduction

1.0. World Malaria Status

Malaria, being one of the world's most important parasitic infections is a major health and developmental challenge for the poor countries of the world (World Malaria Report, 2005). Four species of parasitic protozoa of the genus *Plasmodium* causes malaria in humans: *Plasmodium falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* (Guerin *et al.* 2002). Currently, malaria is identified as an “emerging infection” by many national and international health authorities due to the alarming increase of the disease incidences in many parts of the tropical world (Bremner *et al.* 2001). World Malaria Report (2008) stated that an estimated 3.3 billion people were at risk of malaria in 2006 causing nearly a million deaths, mostly of children less than 5 years while, 247 million episodes of malaria were estimated, with a wide uncertainty interval from 189 to 327 million cases.

1.1. Global status of *Plasmodium vivax* malaria

Plasmodium vivax malaria, which ranges from temperate through the subtropical and tropical zones of the world, exhibits an array of adaptations that enable this parasite to exist in widely varying ecological and climatic conditions (Guerra *et al.* 2006). Malaria caused by *P. vivax* accounts for 25–40% of the 515 million annual cases of malaria worldwide (Carlton *et al.* 2008) and nearly 2.5 billion people, mostly outside Africa, are also at risk from *P. vivax* infections (Herrera *et al.* 2007).

1.2. Epidemiology of malaria in Sri Lanka

Malaria has been a major public health problem and a socio-economic burden in Sri Lanka throughout the twentieth century (Konradsen *et al.* 2000). According to World Health Organization (WHO) report of 2005, during 2000-2005 malaria incidences had shown a dramatic downward trend in the Southeast Asian region, where Sri Lanka has not reported any recent major epidemics since 2000. An estimated population of 10.5 million live in

malaria endemic areas of the island and the annual economic loss due to malaria in Sri Lanka has been estimated at US\$ 15.5 comprising a 6% loss of annual income (Wickremasinghe, 2002). In Sri Lanka, 60 - 80% of malaria cases are due to *P. vivax* (Annual Health Bulletin, 2002). According to the Anti malarial campaign (AMC), a dramatic decrease in *P. vivax* cases were recorded in Sri Lanka during 2000-2007. Nevertheless, in 2008, 93% reported cases were positive for *P. vivax* malaria (Personal communication Dr Galappaththy). Estimating the prevalence of *P. vivax* in districts of Sri Lanka revealed that in 2008 the highest number of *P. vivax* cases were reported from the North and the North East; (in Killinochchi 402 infected out of 670 cases, while 71, 48 and 46 cases were reported from Vavuniya, Mannar and Mullativu, respectively).

In general the degree of endemicity of malaria in different parts of the country has been largely determined by climatic factors which in turn influence the vector breeding potential in an area. Depending on the average annual rainfall, three climatic zones are recognized in the island. Dry zone where the average annual rainfall is less than 2000 mm, the transmission of malaria is increased following monsoonal rains. Malaria remains endemic throughout the region. In the wet zone which experiences an annual rainfall exceeding 2000 mm, malaria is focal and sporadic. Finally, the intermediate zone experiences the unstable type of malaria with increased transmission during dry weather when pooling is seen on river beds and streams (Weekly epidemiological report, 2005).

Monthly parasite incidence patterns of *P. falciparum* and *P. vivax* malaria over the period January 1995 to December 2000, at district resolution was diverse. *P. vivax* generally peaks in January, except for the northern districts, where a second malaria peak of either species occurs around July. This peak is especially pronounced in the west-central districts with very low malaria incidence, where it can even outweigh the January peak. In all districts, *P. falciparum* and *P. vivax* follow approximately the same seasonal pattern, although *P. vivax* seasonality is less explicit due may be to relapses (activation of hypnozoites in the liver). Therefore, the *P. falciparum* incidence seems a better proxy for *P. falciparum* malaria transmission seasonality than *P. vivax* incidence is for *P. vivax* transmission seasonality, although these patterns are also somewhat smoothed by recrudescence (Briët *et al.* 2003).

1.3. Vaccines against malaria

The epidemiology of malaria is characterized by increased prevalence of insecticide resistant vectors and drug resistant parasites resulting in the failure of vector and parasite control measures (Bremner *et al.* 2006). Epidemiological evidence has indicated that following repeated exposure, clinical immunity to malaria is acquired by residents of endemic areas suggesting that the development of a malaria vaccine should be feasible (Greenwood, 2005).

Three principal targets for malaria vaccine(s) would be – i) the pre-erythrocytic stage (sporozoite and hepatic stages), ii) the asexual erythrocytic stage and iii) the sexual or transmission stages.

A pre-erythrocytic vaccine would prevent the entry of sporozoites into hepatocytes and therefore inhibit their development into tissue schizonts, hence would be ideal for *P. vivax* due to the development of hypnozoites, which are responsible for the relapsing nature of the infection (Herrera *et al.* 2007).

Targeting blood stages is effective as antibodies might neutralize the toxic effects associated with schizont rupture that lead to symptomatic paroxysms (the chill and fever associated with schizont rupture) (Karunaweera *et al.* 2003). Vaccines targeting the blood stage may not have to induce sterile immunity (immunity that prevents infection *per se*), which is challenging to achieve, rather effective control of parasite replication may be sufficient to prevent illness and complications, as observed with naturally acquired immunity (Beeson and Crabb, 2007). Antibodies are likely to have a major role in protection through several mechanisms, including inhibition of parasite invasion, intra-erythrocytic parasite blockage and mononuclear cell-mediated inhibition and asexual blood-stage antigens also induce release of cytokines (e.g. IFN- γ) that might contribute to protective immunity. (Reviewed in Herrera *et al.* 2007). Therefore, vaccines designed to target asexual blood stages must induce both antibodies and cytokines.

The sexual or transmission stages are targeted with the aim of producing a vaccine which would prevent sexual stages taken up by a feeding female mosquito from completing their development in the mosquito and thereby preventing the parasite being transmitted to another host, limiting the spread of the parasite (Reviewed in Phillips, 2001).

Development of vaccines for malaria is complicated than anti-viral vaccines due to the complex biology of the parasite, extensive antigenic polymorphism, distinct immune evasion strategies and the ecology of malaria vectors (Richie and Saul, 2002).

1.4. Genetic diversity of *Plasmodium*

The malaria parasite population structure has a significant influence on the gene flow and thus the rate at which new mutations occur which leads to drug resistance or escape from vaccine induced responses. Moreover, this information might give clues on the evolution and selection of pathogens. The studies on population structure quantify how alleles are spatially dispersed and how the overall genetic diversity is organized, hence providing important indications as to the origin, dispersal and stability of multilocus genotypes. Analysis of defined genes provides information about how different alleles are generated and maintained in the population, specifically addressing the relevance of factors such as intragenic recombination and natural selection on the observed polymorphisms (Cui *et al.* 2003). Therefore, studies on malaria parasite population diversity are not only of academic interest to biologists and geneticists, but are of practical importance in the development and deployment of control strategies (Cui *et al.* 2003). The diversity of *P. vivax* was reported in terms of relapse patterns, morphology, biochemistry, drug resistance and genetic diversity (reviewed in Figtree *et al.* 2000). Similar to *P. falciparum*, high levels of polymorphism were apparent for many *P. vivax* genes, particularly antigenic genes that might be under selection by host immunity (Das *et al.* 2007).

1.5. Strain-specific immunity to malaria

The asexual blood stages of malaria parasites are exclusively responsible for the pathology and disease symptoms of malarial infections in human and other vertebrate host species. Protective immunity against these stages is the objective of malaria vaccine research and

development. Exposed individuals living in areas where malaria is endemic can acquire blood-stage protective immunity, however, its extent is highly variable and is dependent, among other features, upon the degree and duration of exposure of an individual to infection with malaria. Conversely, immunity to a challenge infection is readily induced in humans and other animals by clinically induced or laboratory-induced blood infections followed by drug cure when the challenge infection is with parasites of the same stock as the strain used for immunization except, when challenge infections are with parasites of a different genetic origin, albeit of the same species, the degree of protection against the blood parasites and the disease symptoms that they cause is generally significantly less (Reviewed in Cheesman *et al.* 2006).

These observations have led to the concept of “strain-specific” protective immunity in malaria, according to which each naturally experienced inoculum of malaria parasites usually contains a characteristic and genetically determined set of target antigens of protective immunity. Unless containing antigen types previously experienced, each new infection will be poorly protected until all antigen types in the prevailing parasite population have been experienced. This has become the most commonly cited hypothesis to account for the length of time taken to achieve effective protective immunity against malaria in humans exposed to malaria transmission in areas of endemicity (Cheesman *et al.* 2006). However, studies with repeated infections using homologous and heterologous strains in humans have suggested that the immunity raised against *P. vivax* is strain dependant (strain-specific) as well as strain independent (cross reactive / strain-transcending) (Manamperi 2002; Cole-Tobian, 2009).

1.6. Scientific scope of the project (overall and specific objectives):

The consensus is that populations exhibit high levels of genetic polymorphism, most notably the genes encoding blood stage proteins *i.e* Merozoite Surface Protein-1 (*Pvmsp-1*), Apical Membrane Antigen-1 (*ama-1*) and Duffy Binding Protein (*dbp*) which are potential vaccine candidates, show differences in immune recognition ensuing strain-specific immunity (Cole-Tobian and King, 2003; Gunasekera *et al.* 2007; Thakur *et al.* 2008). These findings reflect that the genetic diversity of *Plasmodium* blood stage antigens considered as potential vaccine candidates could be a vital obstacle for the successful development of vaccines against malaria.

Recently our group determined the immuno-epidemiology of the above asexual erythrocytic stage antigens against vivax malaria in patients of Sri Lanka. It was revealed that the humoral responsiveness to these antigens was restricted to approximately 50% among endemic populations and increased with cumulative exposure, implying the existence of strain-specific acquired immunity, due to prevailing local parasite diversity (Wickramarachchi *et al.* 2006 & 2007). Further, Manamperi (2002) recorded that, in Sri Lanka the immune response against the PvMSP-1₄₂ region correlated with the current infection allele, regardless of previous infection. Cole-Tobian and colleagues (2009) demonstrated that children acquire strain-specific antibodies to PvDBPII that can confer better protection against homologous than heterologous strains. Nonetheless, with repeated exposure to multiple PvDBPII variants, individuals acquired immunity to other strains, with a few individuals developing high levels of binding inhibitory antibodies and strain-transcending immunity.

Due to the paucity of information on strain specific immunity to asexual erythrocytic stage antigens (MSP-1, AMA-1 and DBP) to *P. vivax*, the current study was undertaken to examine the prevailing genetic diversity of these antigens in local parasite isolates and to determine the ensuing naturally acquired immune responses (at the isotype level) under prevailing “unstable” malaria conditions in Sri Lanka

Therefore, the overall objective of this study was to determine the ensuing strain-specific immunity to asexual erythrocytic stage (AES) antigens in natural *P. vivax* infections in endemic and non-endemic areas of Sri Lanka.

The specific objectives were:

1. To assess the naturally acquired humoral response (total and isotype specific antibody responses) to asexual erythrocytic stage antigens, i.e. Merozoite Surface Protein -1 (MSP-1), Apical Membrane Antigen-1 (AMA-1) and Duffy Binding Protein (DBP) of *P. vivax* infections of individuals living in endemic and non-endemic areas for vivax malaria in Sri Lanka, using:
 - (a) purified recombinant proteins representing the native MSP-1, AMA-1 and DBP, and

(b) series of overlapping peptides corresponding to MSP-1 (C-terminal hypervariable domain present in p42), AMA-1 (Domain II of the ectodomain) and DBP (critical binding region).

[objective i)(a) has already been achieved through previous NSF grants SIDA/99/BT/01 and NSF/Sch/2004/07, and grant F/3008-1 from IFS, Sweden].

- 2) to characterize the prevailing genetic diversity of *msh-142*, *ama-1DII* and *dbpRII* genes from *P. vivax* isolates collected from infected individuals identified under objective 1.
- 3) to examine the strain-specific immunity to each of the selected AES antigens, MSP-1, AMA-1 and DBP, by aligning the amino acid sequences with the respective total and isotype immune responses.

2.0. Materials and methods

2.1. Ethical considerations and environmental impact

Blood samples were obtained from acute vivax malaria patients, from the National Hospital, Colombo, the Malaria Research Station, Kataragama and the General Hospital, Anuradhapura (N=100 from each study area) for work supported under NSF grant SIDA/BT/99/01 for which ethical clearance was obtained from the University of Colombo, Faculty of Medicine Ethical Review Committee (Ref No. DM/5 (REC-327). Ethical clearance was subsequently sought to use these previously collected blood (serum and parasite) samples (under cold storage), to characterise the ensuing strain specific immunity of the three specified asexual erythrocytic stage antigens of *P. vivax*. This application has received approval of the University of Colombo, Faculty of Medicine Ethical Review Committee (Ref No. EC/04/103) in January, 2005. There was no adverse impact on the environment resulting from the experimental methods used.

2.2. Study sites

Pre-treatment blood samples were collected with informed consent from individuals (age > 15 yrs) acutely infected by *P. vivax* during December 1998 – January 2001, and December 2002 - June 2004 presenting to the General Hospital, Anuradhapura (8°22'N, 80°20'E; N=62), and the Malaria Research station, Kataragama (6°25'N, 81°20'E; N=91), two endemic areas for malaria in the dry zone. Residents of non endemic Colombo in the wet zone, presented to the National Hospital of Sri Lanka (7°55'N, 79°50'E; N=64) had contracted the disease during occupational visits to *P. vivax* endemic areas of the country (Briët *et al.* 2003; Fonseka and Mendis, 1987). Parasitaemia ranged from 0.0001 to 0.025%.

2.3. DNA Extraction

Five ml of blood collected in to a final concentration of 2mM EDTA via vene puncture from *P. vivax* infected patients were separated to divide the serum and the cell pellet. The cell

pellet was re-suspended in 1 x RPMI 1640 medium, and the white blood cells were removed from the medium by passing through a column of CF11 cellulose powder. The effluent from the column was centrifuged and washed twice with RPMI 1640 (Wickramarachchi, 2005). The cell pellet was re-suspended in 1ml of ice cold 1x Natrium Chloride/EDTA/Tris HCL (NET) buffer, pH7.5 (Appendix I) together with 15 µl of 15% solution of Saponin (Sigma Chemicals, USA) and mixed gently. The medium was centrifuged at 3000 x g for 10 minutes and the supernatant was discarded. The pellet was re-suspended in 1ml of 1x NET buffer, and once more was centrifuged at 1000 x g for 10 minutes. The pellet was re-suspended in 0.5 ml 1x NET buffer. To the re-suspension, 5 µl of 10% N-Lauroylsarcosine sodium salt solution and 5 µl of 10 mg/ml ribonuclease A (Sigma Chemicals, USA) was added and incubated for one hour at 37°C. Subsequent to incubation, 5 µl of 20 mg/ml proteinase K (Sigma Chemicals, USA) was mixed and further incubated for 2 hours and 50°C. The mixture was extracted twice with an equal volume of phenol: chloroform: isoamyl alcohol (phenol, 25: chloroform, 24: isoamyl alcohol, 1) (Sigma Chemicals, USA), by centrifuging at 10,000 x g for 10 minutes. The top aqueous layer was carefully pipetted out in to a 1.5 ml eppendorf tubes. The extracted DNA was precipitated by adding twice the volume of ice cold absolute ethanol and 1/10 volume of 3M sodium acetate buffer solution (Sigma Chemicals, USA). The tube containing the suspension was carefully inverted where the DNA could be seen as a small white coil. The DNA containing vial was stored at -70°C for 1 hour. After centrifugation at 13,000 x g for 30 minutes, the supernatant was discarded carefully and the pellet was washed twice with cold 70% ethanol by centrifugation for 10 minutes at 10,000 x g. The DNA pellet was air dried and re-suspended in 50 µl of TE buffer (Appendix II).

2.4. Identification of single clone infections

Parasite stages that infect the human host are haploid and thus, each contains only 1 allele of a given locus from the vivax genome. A patient blood sample may, however, be infected with multiple strains of *P. vivax*. In order to exclude multiple clone infections (MCI) from the present study, each DNA sample was genotyped at the polymorphic *msp-3α* locus using a combined nested polymerase chain reaction (PCR). Amplification of a 1950 bp length of *Pvmsp-3α* was carried out using the following primers.

P1 – 5' CAG CAG ACA CCA TTT AAG G 3'

P2 – 5' CCG TTT GTT GAT TAG TTG C 3'

N1 – 5' GAC CAG TGT GAT ACC ATT AAC C 3'

N2 – 5' ATA CTG GTT CTT CGT CTT CAG G 3'

PCR amplification was performed in a final volume of 50 µl using 1 µl of DNA extract in the preliminary round and 1 µl of the primary reaction in the nested round. Reaction mixture conditions were as follows: 0.5 units of *Taq* polymerase (Promega, USA) was used per reaction, oligonucleotide primers were used at a final concentration of 0.5 µM (Integrated DNA Technologies, USA) and each of the four deoxynucleotide triphosphates at 0.2 mM (Promega, USA). The final MgCl₂ (Promega, USA) concentration was adjusted to 2 mM in the reaction mixture.

The PCR programme was as follows: primary reaction, denaturation at 94°C for 3 minutes, 1 cycle followed by amplification for 30 cycles at 94°C for 30 seconds, 56°C for 30 seconds, and 68°C for 2.5 minutes; nested reaction, 94°C for 30 seconds, 57°C for 30 seconds, and 68°C for 2.5 minutes for 30 cycles. The resultant PCR products were visualized by UV illumination after electrophoresis on 0.8% agarose gels (Promega, USA) containing 0.25 µg/ml of ethidium bromide (Promega, USA, 10 mg/ml). Sizing of products was carried using a standard curve drawn from DNA markers run adjacently (0.1 µg/lane, 1-kb ladder; Promega, USA).

2.4.1. Restriction Fragment Length Polymorphism analysis of *Pvmsp-3a*

The amplified products were subjected to enzyme digestion using *Hha I* and *Alu I* (New England Bio Labs, USA) separately. The following reaction mixtures were made separately for each amplified product:

- i) 0.25 µl *Hha I* enzyme, 2 µl of Buffer 4 (supplied with the enzyme), 4 µl of the PCR product, 0.2 µl of 100 X bovine serum albumin (BSA) (New England Bio Labs, USA) in a final volume of 20 µl.

- ii) 0.5 μ l *Alu I* enzyme, 2 μ l Buffer 2 (supplied with the enzyme), 4 μ l of the PCR product, 0.2 μ l of 100 X BSA in a final volume of 20 μ l.

Both reaction mixtures were mixed thoroughly in separate tubes and incubated at 37°C for 4 to 5.5 hours following incubation at 65°C for 20 minutes. 15 μ l of each digested product was run on a 1.5% agarose gel (Sigma Chemicals, USA) containing 0.5 μ g/ml of ethidium bromide (Promega, USA, 10 mg/ml) at approximately 40-45 volts and visualized under the UV illuminator. Sizing of the digested products was carried using a standard curve drawn from DNA markers run adjacently (0.1 μ g/lane, 100-bp ladder; Promega, USA).

2.5. Amplification of *Pvama-1* domain II

Amplification of a 792 bp length domain II of *Pvama-1* was carried out using the following primers (Gunasekera *et al.* 2007):

2F – 5' GGC AAG GTA TAA AGA CAA TGT AGA G 3'

2R – 5' CAG ATT CAT GTT CCT CGA TTG TTT C 3'

The amplification was carried out in a reaction volume of 50 μ l, using 1 μ l of DNA extract. The reaction mixture included the following: 1 X buffer (Promega, USA), oligonucleotide primers were used at a final concentration of 0.2 μ M (Integrated DNA Technologies, USA) and each of the four deoxynucleotide triphosphates at 0.2 mM (Promega, USA). The final MgCl₂ concentration in the reaction mixture was adjusted to 2 mM (Promega, USA), and the concentration of *Taq* polymerase was 0.5 units (Promega, USA). To avoid formation of primer dimers, a hot start PCR was underwent, where *Taq* polymerase was added to each reaction PCR tube just prior to the annealing step, when the block is heated up to 94°C. The PCR programme for this amplification was as follows: denaturation at 94°C for 5 minutes, followed by amplification for 40 cycles at 94°C for 1 minute, 57°C for 1 minute, and 72°C for 1 minute, the final extension was kept at 72°C for 10 minutes.

2.6. Amplification of *Pvm*sp-1₄₂

Amplification of the 1162 bp length C terminal hypervariable region of *Pvm*sp-1₄₂ was carried out using the following primers (Manamperi, 2002):

P1 – 5' GAT GAC GAC GGG GAG GAA GAC 3'

P2 – 5' AAG CTC CAT GCA CAG GAG G 3'

P3 – 5' GAC CAA GTA ACA ACG GGA G 3'

P4 – 5' GGA CAA GCT TAG GAA GCT GG 3'

PCR amplification was performed in a final volume of 50 µl using 1 µl of DNA extract in the preliminary round and 0.5 µl of the primary reaction in the nested round. Reaction mixture conditions were as follows: 0.5 units of *Taq* polymerase (Promega, USA) was used per reaction, oligonucleotide primers were used at a final concentration of 0.2 µM (Integrated DNA Technologies, USA) and each of the four deoxynucleotide triphosphates at 0.1mM (Promega, USA). The final MgCl₂ (Promega, USA) concentration was adjusted to 2 mM in the reaction mixture.

The PCR programme was as follows: denaturation at 92°C for 2 minutes, 1 cycle followed by amplification for 30 cycles at 91°C for 10 seconds, 59°C for 25 seconds, and 72°C for 25 seconds, and the final extension was at 72°C for 4 minutes. Similar reaction conditions and cycling parameters were used for the nested PCR.

2.7. Amplification of *Pv*dbp region II

Amplification of a 672 bp length region II of *Pv*dbp was carried out by a nested PCR, using the following primers

P1 – 5'GAT AAA ACT GGG GAG GAA AAA GAT 3'

N1 – 5'CTT ATC GGA TTT GAA TTG GTG GC 3'

P2 – 5' GAT CGA AGA TAT CAA TTA TGT A 3'

N2 – 5' TAT CAT AAG GAG TTA CGA TAC 3'.

Four microlitres of the amplified product was visualized under UV illumination after electrophoresis on 1% agarose gels (Promega, USA) containing 0.25 µg/ml of ethidium bromide (Promega, USA, 10 mg/ml) using 2 µl of the PCR product mixed with 1 µl of the Blue/Orange loading dye (Promega, USA). The amplified PCR products were sized using a standard curve drawn from a 100 bp DNA marker run adjacently (Promega, USA).

2.8. Purification and quantification of the PCR products

The domain II *Pvama-1*, *Pvmisp-1₄₂* and *PvdbpII* PCR products were purified according to the manufacturer's instructions using a commercially available kit (Wizard® SV Gel and PCR Clean Up System, Promega, USA). DNA purification was performed by centrifugation and some PCR products containing primer dimers were purified by gel purification. Following electrophoresis the DNA bands were excised and the gel slices were placed in 1.5 ml micro centrifuge tubes. Ten micro liters of membrane binding solution was added per 10 mg of gel slice, vortexed and incubated at 50-65°C until the gel slice was completely dissolved. PCR products without any primer dimers were processed by adding an equal volume of membrane binding solution to the PCR reaction. The dissolved gel mixture or prepared PCR products were transferred to the mini column assembly inserted into the collection tube and incubated at room temperature for one minute. The columns were centrifuged at 16,000 x g for one minute and the flow through was discarded. Seven hundred micro liters of membrane wash solution including ethanol was added, incubated for 2 minutes and centrifuged at 16,000 x g for one minute. The flow through was discarded and the previous step was repeated with 500 µl of the membrane wash solution. The mini columns with the empty collection tubes were re-centrifuged at 16,000 x g for 5 minutes to evaporate any remaining ethanol. Mini columns were carefully transferred to new 1.5 ml centrifuge tubes and 50 µl of nuclease free water was added, incubated for a further two minutes at room temperature and centrifuged at

16,000 x g for one minute. The mini columns were discarded and the eluted DNA was stored at 4°C or -20°C for further use.

Two micro liters of the purified DNA mixed with 1 µl of the tracking dye were visualized under UV illumination after running on 1% agarose gels (Promega, USA) containing 0.25 µg/ml ethidium bromide (Sigma Chemicals, USA, 10 mg/ml). The intensity of the PCR products were compared with known molecular standards (Low mass DNA ladder, Invitrogen, USA) to quantify the DNA amount contained.

2.9. Sequencing of the purified PCR products

Sequencing of each of the purified PCR products of domain II *Pvama-1*, *Pvmosp-1₄₂* and *PvdbpII* genes were carried out by Macrogen, Korea. The final concentration was adjusted to 30 – 50 ng/µl with a volume of 20-50 µl prior to direct sequencing. Nested primers were used to sequence the *Pvmosp-1₄₂* and *PvdbpII*. Four-fold coverage was attempted for at least 10 isolates for each of the three loci.

2.10. Data Analysis

Sequences were aligned and verified using Seqman II (DNASTar, Madison, WI) and consensus sequences were aligned using ClustalW of the MEGA 4.1 software. The sequence data of each gene were submitted to the Gene Bank database under the accession numbers GU175174 – GU175268 for *Pvmosp-1₄₂*, GU433899 - GU433939 for *Pvama-1* domain II and GU143914 - GU144013 for *PvdbpRII*. DnaSP version 4.50 (Rozas *et al.* 2003) was used for population genetic studies, while MEGA 4.1 (Tamura *et al.* 2007) was used to construct the phylogenetic trees by using the Kimura 2 parameter and 1000 bootstrap pseudo-samples and the numbers of synonymous nucleotide substitutions per synonymous site (Ds), the number of non-synonymous substitutions per non-synonymous site (Dn), and the difference between the non-synonymous and synonymous substitutions (Dn-Ds) were estimated by Nei and Gojobori's method.

Using a T-cell epitope-prediction algorithm (SYFPEITHI software) (Rammensee *et al.* 1999) and ProPred MHC class II binding peptide prediction server (Singh and Raghava, 2001), the

prediction of differential bindings of epitopes on PvMSP-1₄₂, PvAMA-1DII and PvDBPRII to class II MHC allotypes were performed. Specifically MHC alleles HLA-DRB1*0101, DRB1*0401, DRB1*0701 and DRB1*1101 were selected as these four alleles were among the 10 most prevalent in Afro-American and Asian populations, (Southwood *et al.* 1998).

Using the BcPred software (EL-Manzalawy *et al.* 2008), linear B cell epitopes spanning the 33 kDa region of PvMSP-1₄₂, PvAMA-1DII, and PvDBPRII was predicted.

2.11. Designing the series of overlapping peptides to PvAMA-1 domain II

To evaluate the preferential recognition of linear B cell epitopes spanning the 136 amino acid region of domain II PvAMA-1 (corresponding to amino acid 207 – 343 of the PvAMA-1 Salvador I strain), seven synthetic peptides (P01-P07) were designed with overlapping 4-6 amino acids. The designed peptides were synthesized by Mimotopes Pty Ltd, Australia.

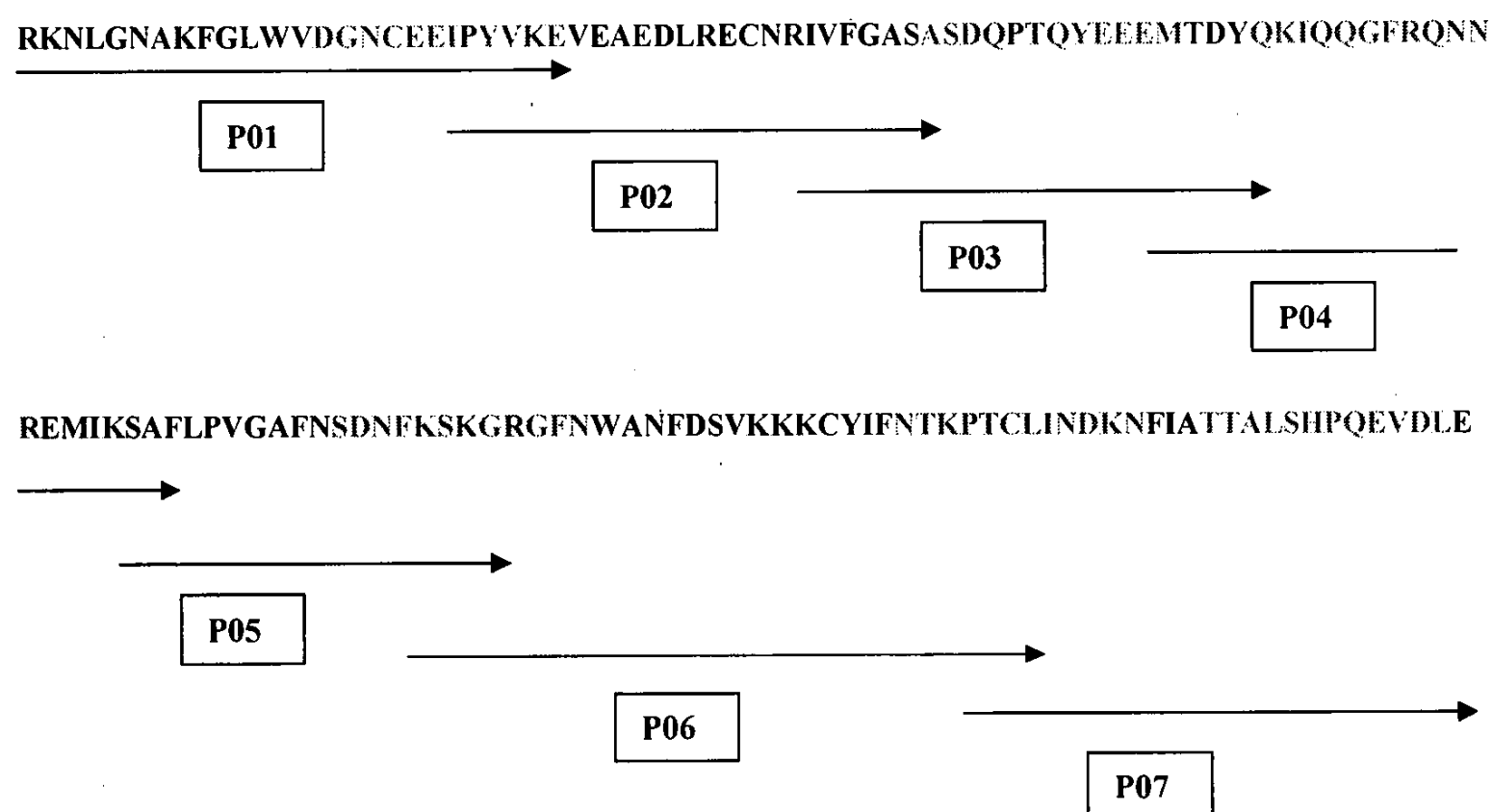


Figure 1. Schematic representation of the seven synthetic peptides designed to PvAMA-1 domain II

Predicted linear B cell epitopes are depicted in blue.

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

2.12.1. Serum

The serum samples (N=162) from the three study areas that were positive with total (IgG + IgM) antibody response to the recombinant PvAMA-1 ectodomain (Wickramarachchi, 2005) were selected as test samples for this study. Healthy residents of Colombo (N=30) who were never exposed to malaria served as controls.

2.12.2. Determination of anti-PvAMA-1 domain II antibodies

The total (IgG + IgM) antibody responses to the seven synthetic overlapping peptides were assayed by an indirect micro plate enzyme linked immunosorbant assay (ELISA) as previously described (Wickramarachchi, 2005) with modifications. Each test serum sample was assayed against each peptide in duplicate with normal human serum as control. Checkerboard titrations determined the optimal dilutions of reagents used in this ELISA.

Hundred micro liters of each peptide (in appropriate concentration) mixed in 1X phosphate buffer (PB), pH 7.4 (Appendix III) was used to coat the surface of 96 wells microtitre flat bottom plates (Immunol 2HB, Dynatech Laboratories, USA). Wells at the edge of the plates were avoided to overcome edge effect. The plates were incubated over night at 4°C. The contents of the wells were aspirated and each well was blocked with 200µl of blocking buffer (Appendix IV). Plates were incubated at room temperature for two hours in a humid chamber and flick washed four times in washing buffer (Appendix Va) and the plates were air dried by means of downward thrust against a tissue pad. Serum samples were diluted according to each peptide in dilution buffer (Appendix VIa) and 100 µl were added to each of the duplicate wells. The plate was incubated for two hours at 37°C and washed eight times in washing buffer. Suitable dilutions of the HRP linked rabbit anti-human IgG and IgM antibodies were prepared freshly in dilution buffer and 100 µl was dispensed to each of the wells and incubated for one hour at 37°C. After washing four times in washing buffer, 200 µl

of substrate OPD (O-phenylenediamine) (Sigma Chemicals, USA) at a concentration of 1 mg/ml in substrate buffer (Appendix VII) with 0.03% hydrogen peroxide (H₂O₂) (Sigma Chemicals, USA), were dispersed to each well. The colour reaction was allowed to proceed for 15 minutes at room temperature in the dark and the reaction was arrested with 50 µl of 2 N sulphuric acid (Appendix VIII), after which the optical density (OD) was measured at 490 nm in an automated ELISA reader (Model 680, BioRad International, USA).

The cut-off value for this test was calculated to be the mean OD value plus two standard deviations of the normal controls. Mean OD values obtained for test samples falling over and above this cut-off level were expressed as positive responses.

2.12.3. Isotype determination

As the total antibody responses to each of the seven peptides of PvAMA-1 domain II in the study areas revealed that individuals tend to respond more to peptides P04 and P05, these two peptides were further subjected to the analysis of isotype specific IgM and IgG subclasses antibody responses. Checkerboard titrations determined the optimal dilutions of reagents used in each isotype determination. Serum samples of responders with total antibodies to PvAMA1 domain II P04 and P05, from each test area (Colombo, N=36; Anuradhapura N=33, and Kataragama, N=65) were selected for this study. Each test serum sample for each isotype was assayed against the protein in duplicate with normal human serum (N=30) as control.

To measure IgM and IgG subclasses peptides P04 and P05 were bound to surfaces of 96 well microtitre plates (Immunol 2HB, VWR International, UK). Wells were coated each with 50 µl of the antigen in appropriate concentration in 1X PB, pH 7.4 and were kept overnight at 4°C. The wells were aspirated and blocked with 100 µl of blocking buffer and incubated for two hours at room temperature in a humid chamber. The plates were flick washed four times, air dried and 50 µl of this sample at a dilution of 1:15 in dilution buffer was dispensed to duplicate wells. The plates were incubated for two hours at 37°C and washed eight times. To each well, 50 µl of isotype specific mouse anti-human Mabs at the appropriate dilution was added and incubated overnight at room temperature. The plates were washed eight times, and affinity purified HRP linked goat anti-mouse IgG (Sigma Chemicals, USA) in appropriate dilutions were added to each well and incubated at 37°C for one hour. After washing four

times, 100µl of substrate OPD with 0.03% H₂O₂ was added to each well. The colour reaction was allowed to proceed for 30 minutes at room temperature in the dark and the reaction was arrested with 50 µl of 2N sulphuric acid, after which the optical density (OD) was measured at 490 nm in an automated ELISA reader (Model 680, BioRad International, USA).

The cut-off value for this test was calculated to be the mean OD value plus two standard deviations of the normal controls. Mean OD values obtained for test samples falling over and above this cut-off level were expressed as positive responses.

2.12.4. Statistical analysis

Statistical analysis was performed using SPSS version 15 for windows (SPSS Inc, USA) and Epi Info 6 (version 6.04b to c upgrade) (CDC, USA and World Health Organization, Switzerland) computer software packages. Proportion of responders (total and isotype specific) using independent variables were tested using the Chi squared test. Normally distributed independent variables were compared using the T-test, ANOVA while not normal distributions were tested using Mann-Whitney U test and the Kruskal-Wallis test. Dependable variables, normal or not normal distribution were tested using the paired T test and Wilcoxon Signed Rank test. In order to develop *in vivo* correlates of protection attempts were made to sort associations between antibody responses and host factors by using bivariate correlations (Spearman and Pearson correlation coefficients), and Chi square for linear trend as appropriate. The significant level was set at P<0.05.

2.13. ELISA based binding inhibition assay

An ELISA based binding assay was developed by Hans *et al.* (2005), to study the specific binding of recombinant PvRII to its cognate receptor on erythrocytes, the Duffy blood group antigen (DA). This assay was further modified as an ELISA based binding inhibition assay, and was used to assess the functional ability of the naturally acquired antibodies to block the specific binding of PvRII with human DA receptor.

Ninety-six well microtitre flat bottom plates (Nunc, Maxisorp, USA) were coated with 1µg/ml of recombinant nDA-Ig in 100µl of coating buffer (Sodium bicarbonate- pH 9.6)

(Appendix IX) and incubated overnight at +4°C. Plates were washed 3 times with washing buffer (Appendix Vb), with a 1 minute wash each using an ELISA plate washer (BIORAD, USA). Unoccupied binding sites of each well were blocked with 200 µl of blocking buffer (Appendix IVb) for 2 hrs at 37°C in a humid chamber.

While the plate was being blocked, recombinant protein PvRII was pre incubated with each of the test serum samples for 1hr at 37°C in stationary condition. Final concentration of the PvRII was adjusted as 0.1 µg/ml while each of the serum samples was adjusted in a dilution series of 1:5, 1:10, 1:20, 1:50 and 1:100 in dilution buffer (Appendix VIb). Pre incubation of serum and PvRII was carried out for 1hr at 37°C in stationary condition.

After blocking for 2hrs, ELISA plates were washed and 100 µl of the pre incubated PvRII-test serum mixture was added to the wells in duplicate for each serum dilution; and further incubated for 1 hr 37°C. After washing as previously indicated, 100 µl of anti-PvRII rabbit polyclonal serum containing antibodies against recombinant protein PvRII (ICGEB, India) at a dilution of 1:8,000 in dilution buffer (VIb) were added to wells and was incubated for 1 hr at 37°C and then plates were washed as previously. Plates were incubated with anti-rabbit HRP conjugate (Sigma, USA) at a dilution of 1: 5000, at 100 µl per well and incubated for 1 hr at 37°C. Finally plates were washed 3 times with washing buffer (b) followed by washing twice with 1XPBS. After plates were dried, 50µl of substrate buffer including o-Phenylenediamine (OPD) (Sigma,USA – Product No: P9029) at a final concentration of 1µg/ml and 0.1% of H₂O₂, was dispensed to each well. The colour reaction was allowed to proceed for 10 minutes and was stopped by adding 25 µl/well of 2NH₂SO₄. The optical density (OD) at 492nm was measured in an automated ELISA reader (BIORAD, USA).

For each plate, pooled test serum (consisting of 15 serum samples) and mouse pre-immune anti-PvRII serum were used, at a dilution of 1:10 as positive controls. Pooled serum from 20 normal healthy controls served as negative controls. Further, for validation of each assay, recombinant mutant type YF-nDA-Ig was tested for the binding with PvRII. For each plate, OD values in duplicate were obtained for direct binding of nDA-Ig with PvRII (without incubating PvRII with sera).

For each plate, the mean OD from duplicate values resulting from the direct binding of nDA-Ig with PvRII, was considered as the OD that represented 100% binding. The OD values

obtained with the presence of test serum at each dilution was normalized by expressing as a percentage of OD at 100% binding.

$$\% \text{ Binding with the presence of test serum} = \frac{\text{Mean OD of the test serum}}{\text{OD at the direct binding of nDA-Ig and PvRII}} \times 100$$

For each test serum, a curve was plotted for % binding against each dilution. Using these graphs, 50% binding titer was obtained for each test serum. The 50% binding inhibition titer and the level of inhibition at each serum dilution was also calculated for each test serum.

The mean % inhibition was calculated for each serum dilution tested of the 20 normal healthy control sera samples. Serum samples with the ability to inhibit the binding of PvRII to nDA-Ig at particular serum dilution were selected as those that exhibited a higher level of inhibition than that of normal healthy controls.

2.14. Strain-specific immunity to each protein

To evaluate possible strain specific immune responses against domain II of PvAMA-1, fifty two of the 64 *P. vivax* AMA-1 domain II amino acid sequences were aligned with the homologous total (IgM + IgG) anti-PvAMA-1 antibody responses assayed by an indirect ELISA against seven synthetic peptides of PvAMA-1 domain II. The seven synthetic peptides resembled the native Salvador I strain.

To evaluate possible strain specific immune responses against PvMSP-1₄₂, seventy two of the 95 *P. vivax* MSP-1₄₂ amino acid sequences, representing 25 of the 27 amino acid haplotypes (as haplotypes H14 and H23 lacked homologous serum samples for analysis) were aligned with the homologous total (IgM+IgG) anti-PvMSP-1₄₂ and PvMSP-1₁₉ antibody responses previously assayed by an indirect ELISA against recombinant protein PvMSP-1₄₂ (Wickramarachchi, 2005). The recombinant protein PvMSP-1p42 expressed in the baculovirus system resembled the native Belem construct (Longacre *et al.* 1994; Holm *et al.* 1997). The same analysis was repeated for PvMSP-1₁₉.

The nature of the naturally acquired anti-PvDBPII antibodies were elucidated with respect to the existing variant PvDBPII haplotypes / strains circulating among the field parasite isolates in Sri Lanka.

Results

Results are presented under 4 separate chapters (3-6) with relevant discussion of the results under each chapter. The diversity of PvMSP-3 α locus and the PvMSP-1 are presented under chapters 3 and 4 respectively. Chapters 5 and 6, deal with both genetic diversity and strain specific-immunity to PvAMA-1DII and DBPIIR, respectively.

Chapter 3. Genetic Diversity of the *Pvmsp-3α* locus

3.1. Identification of *P. vivax* single clone isolates

3.1.1. Introduction

Malarial parasites are haploid except during a brief period of sexual recombination in the mosquito vector. The infection of a single mosquito with multiple strains of *Plasmodium* results from the mosquito feeding either on a single host that carries more than one parasite genotype or, as the result of interrupted feeding, on multiple hosts within one gonotrophic cycle (Druilhe *et al.* 1998). In humans, multiple-clone infections (MCI) with malarial parasites may result not only from a single bite of a mosquito that has a multiple-clone infection itself but also from relapse infections and super infections in the human hosts (Havryliuk and Ferreira, 2009). The degree of genetic heterogeneity seen in both *P. vivax* and *P. falciparum* infections in humans has been associated with the prevailing pattern and intensity of transmission in the study area (Bruce *et al.* 1999, 2000; Engelbrecht *et al.* 2000; Magesa *et al.* 2002).

The genotyping of *P. vivax* infections has been based on several polymorphic antigens (Premawansa *et al.* 1993; Kolakovich *et al.* 1996; Bruce *et al.* 1999, 2000; Cui *et al.* 2003; Cole-Tobian *et al.* 2005). The gene coding for the parasite's merozoite surface protein-3α (*msp-3α*) appears to be a particularly reliable marker for the molecular epidemiology of *P. vivax* in various geographical areas, having a central polymorphic region that is readily evaluated by use of a combination of PCR and RFLP (Bruce *et al.* 1999, 2000; Galinski *et al.* 1999; Cui *et al.* 2003; Cole-Tobian *et al.* 2005; Véron *et al.* 2009). The complexity of the *P. vivax* infections in Sri Lanka has previously been demonstrated in a few small-scale studies based on various markers and tools (Langsley *et al.* 1988; Udagama *et al.* 1990; Premawansa *et al.* 1993; Gunasekera *et al.* 2007; Karunaweera *et al.* 2008; Manamperi *et al.* 2008).

3.2. Objective of the study

The present study was carried out to separate single clone isolates from the multiple clone isolates and to subsequently analyse the genetic complexity of *P. vivax*, in clinical isolates of Sri Lanka, using the *msp-3α* locus as a molecular epidemiological marker.

3.3. Results

3.3.1. Characteristics of the test populations

Although 90% of the subjects from the endemic areas presented (for malaria diagnosis and treatment) within 4 days of the onset of their symptoms, only 50% of the subjects from non-endemic Colombo had sought treatment within 6 days of becoming symptomatic. The mean ages of the subjects from the three study areas were similar ($P>0.05$ for each comparison) but the subjects from Kataragama reported a significantly higher number of previous malarial attacks than the subjects from Anuradhapura or Colombo, with means of eight, three and two, respectively ($P<0.05$). The subjects from Anuradhapura, Kataragama and Colombo presented with mean parasitaemia of 0.1%, 0.07% and 0.14% respectively, the values for Kataragama and Colombo being significantly different ($P<0.05$). Although half of the subjects from Anuradhapura had had a malarial attack in the previous 5 months and half of those from Kataragama had had such an attack in the previous 3 months, most of the subjects from Colombo were suffering from their first attack of malaria.

3.3.2. Prevalence of multiple-clone *Plasmodium vivax* infections

Of a total of 218 *P. vivax* clinical isolates, 201 were successfully amplified at the *msp-3α* locus. Evaluation of MCIs in the three study areas revealed 5 isolates with more than one band in the undigested PCR products. Further, the summation of RFLP fragments in 32 isolates resulted in significantly greater values than the corresponding undigested product, indicating the presence of more than one *Pvmsp-3α* allele in these infections (Table 3.1.1). The total number of multiple clone infections from malaria endemic areas were 19.2% and 20.8% from Anuradhapura and Kataragama-Buttala, respectively, compared with 13.8% from non-endemic Colombo. Though not significant, the number of these MCIs were collectively

higher in the two endemic areas (20%) compared to the non-endemic area (Chi-square test, $P>0.05$).

Table 3.1. Number of multiple clone infections determined from the three study areas

Study area	Number of samples	Number of multiple clone infections ^a				
		PCR ^b N (%)	PCR/RFLP			Overall ^c N (%)
			<i>Hha</i> I only N (%)	<i>Alu</i> I only N (%)	Both <i>Hha</i> I & <i>Alu</i> I N (%)	
<u>Endemic</u>						
Anuradhapura	52	1	3	4	2	10 (19.2%)
Kataragama	91	3	8	7	1	19 (20.8%)
<u>Non-endemic</u>						
Colombo	58	1	1	4	2	8 (13.8%)
Total	201	5 (2.5%)	12 (6%)	15 (7.5%)	5 (2.5%)	37 (18.4%)

^a Total number of clinical isolates analysed by PCR = 201, and PCR/RFLP = 196

^b Multiple bands from nested PCR

^c The total number of multiple clone infections detected by nested PCR, and either by one of the two RFLP patterns obtained with the two enzymes *Hha* I and *Alu* I or by both.

3.3.3. Genetic diversity of *Pvmsp-3a*

3.3.3.1. Prevalence of different alleles of *msh-3a*

Subsequently, further analysis at this locus exposed the genetic complexity of *P. vivax* in Sri Lanka, where based on the size of the undigested PCR products, three sizes of 1900, 1500 and 1200 bp were identified. The predominance of the 1900 bp product (N=146, 72.6%) was significant than the other two products of 1500 (N=52, 25.9%) and 1200 bp (N=3, 1.5%) in all three study areas [Chi square test: (with 1500 bp; P<0.05) and (with 1200 bp; P<0.01)]. As indicated previously, PCR amplifications resulted in multiple bands (1900 and 1500 bp) in 5 samples (Anuradhapura: N=1, Kataragama-Buttala: N=3 and Colombo: N=1) providing prime evidence for multiple clone infections (Table 3.1).

3.3.3.2. Genotypes Identified by PCR-RFLP

The RFLP patterns of all isolates showed size conservation of the largest fragment obtained from *Hha* I digestion (~1,000 bp) and *Alu* I digestion (~500 bp), while smaller fragments that were used to distinguish different genotypes showed considerable size variation ranging from 175 to 600 bp for *Hha* I, and 150 to 450 bp for *Alu* I. The RFLP banding patterns of 196 samples resulted in 22 different genotypes with *Hha* I digestion, while 26 were recorded for *Alu* I. The diverse genotypes were mostly confined to the 1900 bp allele. The four genotypes from each digestion, H1 - H4 from *Hha* I and A1 - A4 from *Alu* I, represented more than 50% of infections analyzed by PCR/RFLP. The combined *Hha* I and *Alu* I patterns resulted in 51 different genotype combinations.

The distribution of different genotypes from restriction digestion showed uneven distribution between the two endemic areas, suggesting that some of these may be prominent in either one of the endemic settings. Genotypes H1, H2 and H3 were restricted to isolates sampled from Kataragama-Buttala, while H6, H7 and H8 were exclusively found in Anuradhapura. It is noteworthy that these genotypes restricted to one of the two endemic areas were among the most predominant types and accounted for a majority of infections sampled from that particular endemic area (Anuradhapura: 80.5%; Kataragama-Buttala: 86.3%). Though less

significant compared to *Hha* I genotypes, some genotypes resulted from *Alu* I digestion also showed uneven distribution between the two endemic areas *i.e.*: A2, A4 and A7 (Figure 3.1).

3.3.3.3. Genetic diversity of *Plasmodium vivax msp-3α* in Sri Lanka

Direct sequences of 17 single-clone infections representing 1900 bp (N=8), 1500 bp (N=8) and 1200 bp (N=1), different alleles of *msp-3α*, were readable only between amino acid positions 112 and 662 derived according to the *Pvmsp-3α* Belem strain (Rayner *et al.* 2002). While 196 polymorphic sites in the 1900 bp genotype (N = 8) gave rise to 202 mutations, the 1500 bp genotype (N = 8) with 5 polymorphic sites resulted in only 5 mutations, demonstrating the limited polymorphism in the latter, due to the deletion within the hyper-variable region of block I. Interestingly, the 1900 bp genotype comprised of 8 different haplotypes, resulting in high haplotype diversity ($H_d = 1$) whereas only 05 haplotypes resulted from the 1500 bp allele type ($H_d = 0.786$). Phylogenetic analysis showed that isolates with the 1900 bp allele were not clustered into a specific clade but distributed throughout the tree forming 3 clades, while 1500 bp isolates robustly clustered into a single sub group of a clade (Figure 3.2).

Though PCR/RFLP data of these 17 isolates produced only 11 different haplotypes, the corresponding sequence data resulted in 13 distinct haplotypes indicating more diversity at the nucleotide level.

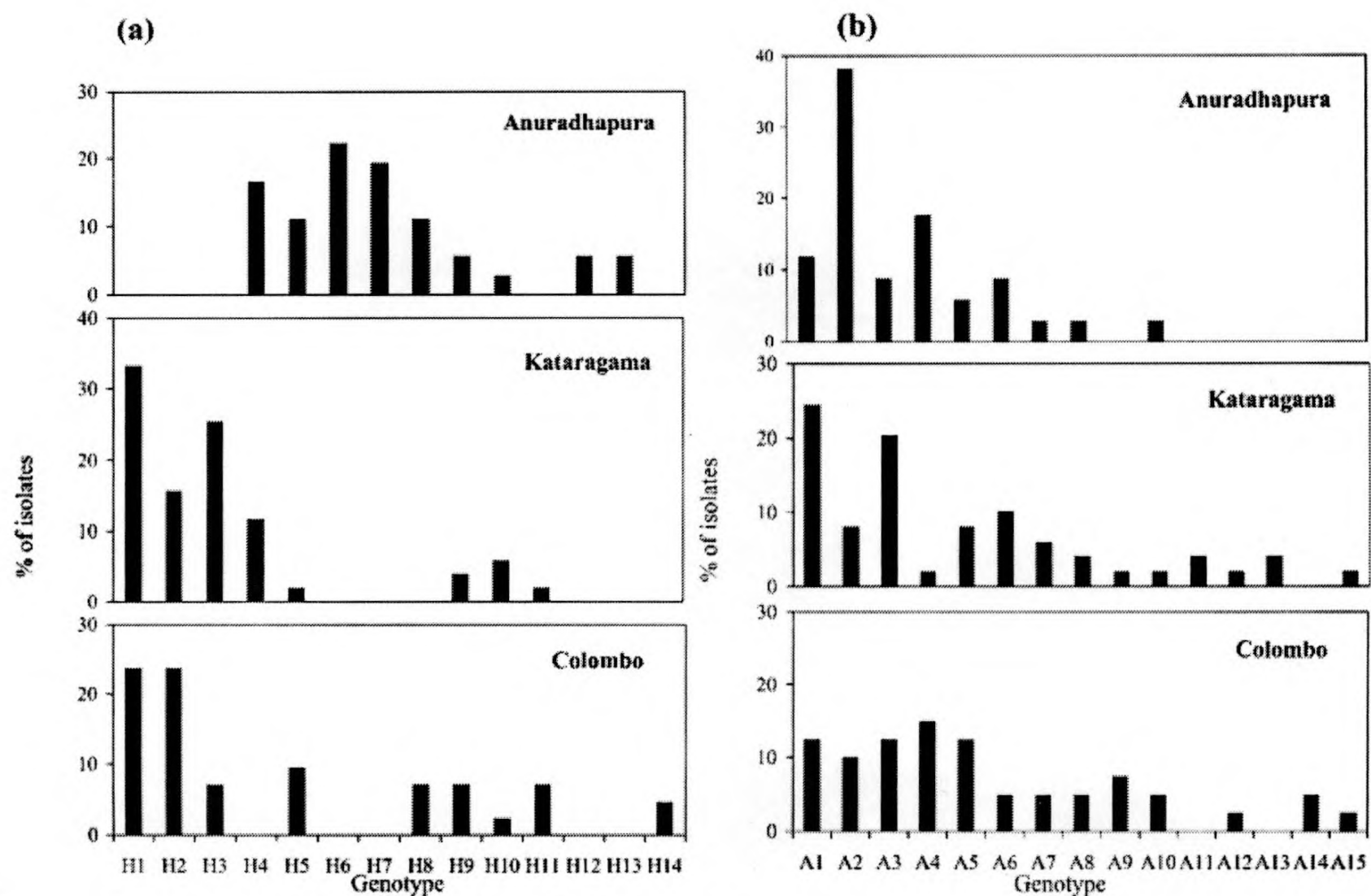


Figure 3.1. Some of the results of the PCR-RFLP genotypes, showing the percentages of the single-clone isolates from each of the three sample-collection sites identified as (a) genotypes H1-H14 (after HhaI digestion) or (b) genotypes A1-A15 (after AluI digestion).

Only the data for genotypes that each represented 1% of the single- clone isolates with 1900-bp alleles are shown.

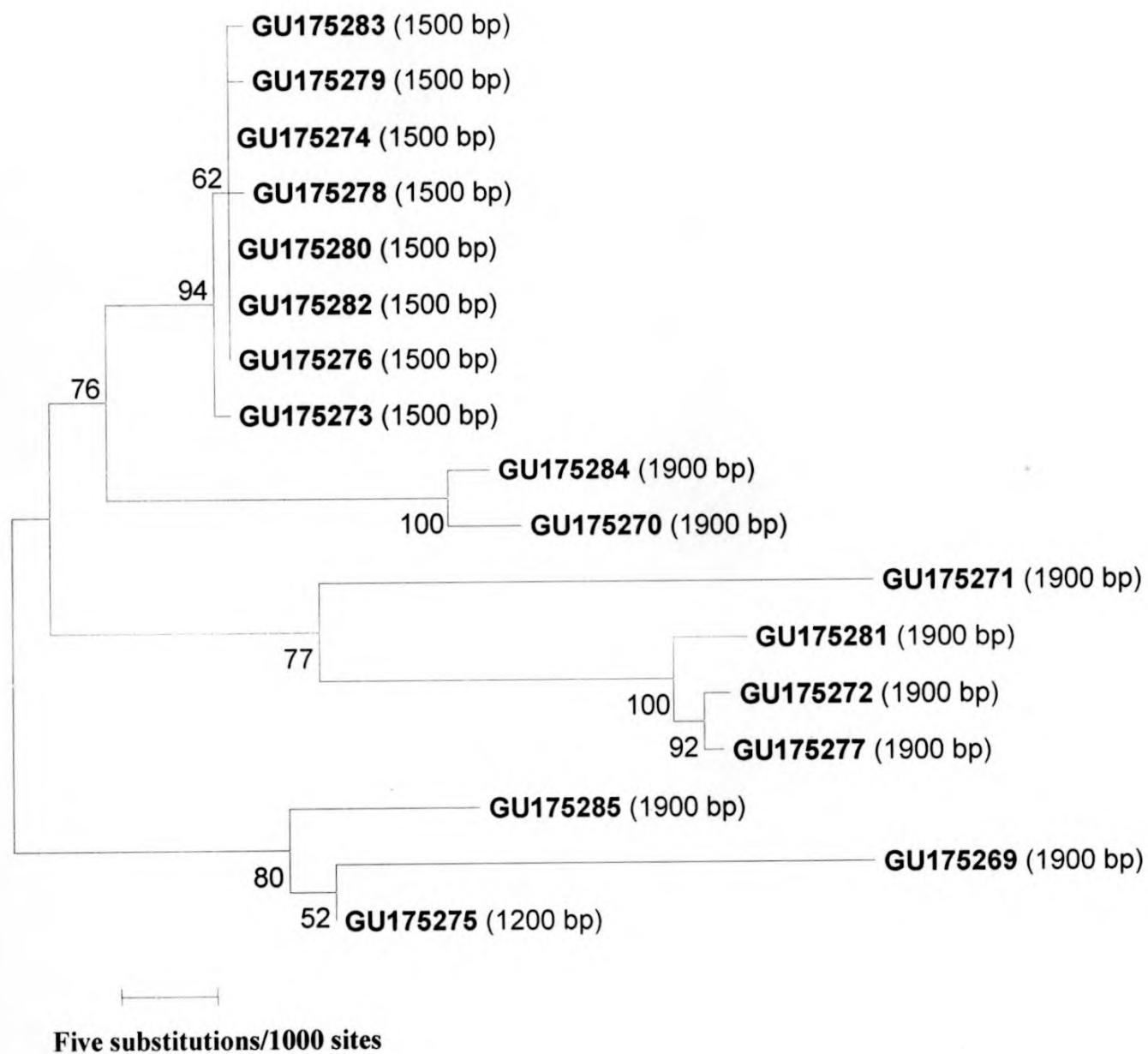


Figure 3.2. Phylogenetic tree based on the *msp-3α* genes of 17 Sri Lankan isolates of *Plasmodium vivax*

The tree was constructed using the neighbour-joining method. The *msp-3α* alleles investigated were represented by 1900-, 1500- or 1200-bp amplicons in the PCR. Each isolate number on the phylogenetic tree is the relevant GenBank accession number.

3.4. Discussion

The close association of genetic complexity of malaria infections and prevalence of MCIs with transmission intensity of that locality is well established (Babiker *et al.* 1997; Anderson *et al.* 2000; Cristiano *et al.* 2008). This is the first molecular epidemiology report on the genetic complexity of *P. vivax* infections with a significant number (N=201) of clinical isolates sampled from both malaria endemic and non-endemic areas in Sri Lanka. The presence of 20% multiple clone infections (MCIs), prevalence of size polymorphism and diverse genotypes at the *msp-3 α* locus indicate the degree of genetic complexity of local *P. vivax* infections, in an endemic setting with low and unstable malaria transmission (Rajendram and Jayewickreme, 1951; Mendis *et al.* 1990). In the present study, we report a higher MCI (20%) via genotyping of the *Pvmsp-3 α* locus, a reliable molecular epidemiologic marker, by combined PCR/RFLP (Kim *et al.* 2006; Zakeri *et al.* 2006; Cristiano *et al.* 2008; Véron *et al.* 2009). Thus, this could be more representative of the local situation, and may be directly compared with MCI rates in other endemic countries with different malaria transmission intensities than Sri Lanka. Our findings are comparable to those of Bruce & others (1999) from several geographic areas hypo-endemic to malaria transmission, including Thailand and Venezuela (Cui *et al.* 2003; Ord *et al.* 2005).

Three basic factors may be responsible for multiple clone infection; *i.e.* co-infection, super infection and somatic mutation during the mosquito infection (Cui *et al.* 2003). Residents of both endemic as well as non-endemic areas have an equal probability to receive an infectious mosquito bite with either a co-infection or a somatic mutation. Measured entomological inoculation rates in Kataragama describe that only 1/500 individuals have the chance to receive an infectious mosquito bite per night (Mendis *et al.* 1990). Thus, super infections may be rare due to the low malaria transmission intensity in the endemic areas, as such an event may be possible only with a lag period in between the primary and secondary *P. vivax* infectious bites.

We, for the first time report geographic distribution of *P. vivax* genotypes in Sri Lanka. The uneven distribution of some of the PCR/RFLP genotypes between the two malaria endemic areas situated 250 km apart is plausible, due to the prevailing low transmission intensity and the lower MCI prevalence (Anderson *et al.* 2000). As a result, unrelated parasites rarely co-

exist in the same mosquito blood meal, which in turn leads to parasite inbreeding. Presence of geographic distribution due to inbreeding has been previously observed in areas with low transmission intensity *i.e.* South America, whereas out breeding was reported in high transmission areas *i.e.* Africa (Anderson *et al.* 2000). The appearance of these geographically restricted genotypes in infections sampled from the local non-endemic area indicated a recent visit to a particular endemic area that was further supported by verbal confirmation from respective patients during sample collection.

Pair wise nucleotide diversity (π) obtained from different merozoite proteins of *P. vivax* using the same battery of local clinical isolates as the current study indicated that the value of *Pvmsp-3 α* ($\pi = 0.059$) is the highest among these (*PvdbpII*: $\pi = 0.0098$, *Pvama-1DII*: $\pi = 0.0095$ and *Pvmsp-1₄₂*: $\pi = 0.0231$) (Udagama-Randeniya, unpublished data), which also is comparable with previous reports on *Pvmsp-3 α* (Mascorro *et al.* 2005). The overall haplotype diversity (Hd) recorded for the 1900 bp fragment signify the high intensity of genetic diversity of the *Pvmsp-3 α* locus of parasites circulating in the local endemic setting. The high pair wise nucleotide diversity, subsequent haplotype diversity and high prevalence of the 1900 bp allele reiterate the suitability of *Pvmsp-3 α* gene as a genetic marker to evaluate genetic complexity of *P. vivax* infections in Sri Lanka. Moreover, the low fixation index value ($F_{ST} = -0.135$) demonstrating a genetic drift between isolates of Anuradhapura and Kataragama corresponding to 1900bp, reiterated the purifying selection observed at the entire 1900bp gene of *Pvmsp-3 α* . Further, the established geographic distribution of genotypes detected in the two endemic areas may most likely result from the genetic drift observed between these two areas. Since the phylogenetic analysis showed that the 1900 bp allele formed 3 clades that were distributed throughout the tree, while 1500 bp allele was restricted to a single sub group of a clade may indicate that the 1900 bp version is the ancestral form and has had far longer to diverge, whereas the 1500 and 1200 are derived forms and evolved more recently.

In conclusion, these results indicate that the overall MCI prevalence was 20% in *P. vivax* endemic infections in Sri Lanka with low and unstable malaria transmission settings where patients present for early diagnosis and treatment. The higher MCI prevalence in endemic *P. vivax* infections compared to infections sampled from the non-endemic area suggests an impact of relapses and super infections on MCIs. The prevailing genetic diversity, the proportion of multiple clone infections and the presence of distinct genotypes in different

endemic areas in the island highlight the degree of complexity of *P. vivax* infections in Sri Lanka.

Chapter 4. Genetic diversity of *Plasmodium vivax* Merozoite Surface Protein-1₄₂ (*Pvmsp-1*₄₂) in Sri Lanka

4.1 Introduction

Surface components of the infected erythrocyte and invasive merozoite stage of the parasites have therefore been studied intensively for their ability to induce protective immune responses (José *et al.* 1997). Ten members of the Merozoite Surface Protein (MSP) family have been described in *P. falciparum* (*Pfmsp1-10*) of which, eight have been recognized in *P. vivax* (*Pvmsp-1*, *Pvmsp-2*, *Pvmsp-3α*, *Pvmsp-3β*, *Pvmsp3γ*, *Pvmsp-4*, *Pvmsp-5*, *Pvmsp-8*, *Pvmsp-9*, and *Pvmsp-10*) (del Portello *et al.* 1991; Barnwell *et al.* 1999; Galinski *et al.* 1999; Black *et al.* 2002; Vargas-Serrato *et al.* 2002; Perez-Leal *et al.* 2004 and 2005).

Several subunit malaria vaccines in clinical development are based on Merozoite Surface Protein 1 (MSP-1), the major protein on the surface of the extracellular blood stage of the parasite (Takala *et al.* 2007). Present abundantly on the surface of mature merozoites, Merozoite Surface Protein 1 (MSP-1) is expressed initially as a 200-kDa protein during the late stages of erythrocytic schizogony (John *et al.* 2004). A required component of the merozoite invasion machinery, MSP-1 associates non-covalently with other parasite proteins, including the C-terminal portion of MSP-6 (Trucco *et al.* 2001). To date, only two complete *Pvmsp-1* sequences are available from two monkey-adapted strains of *P. vivax*, Brazilian Belem and Central American Salvador I strains (del Portillo *et al.* 1991; Gibson *et al.* 1992). The Belem strain can easily be characterized by a variable poly-glutamine (poly Q) region which is absent in the Salvador I strain (Razavi *et al.* 2006).

As a requirement for merozoite entry into a red cell, MSP-1 undergoes two processing steps, initially during merozoite release from an infected cell followed by invasion of a red cell (Holder *et al.* 1992). In the first processing step, the precursor polypeptide is cleaved into four chains (with apparent molecular masses of 83, 30, 38, and 42 kDa) held together by non-covalent contacts on the surface through the glycosylphosphatidylinositol GPI anchor on the C-terminal 42 kDa fragment, MSP-1₄₂ (Holder *et al.* 1992). During erythrocyte invasion secondary cleavage of MSP-1₄₂ leads to shedding of the complex, that generates a 33 kDa fragment (MSP-1₃₃), derived from the N terminus of MSP-1₄₂ (Blackman *et al.* 1991), while MSP-1₁₉, the second product of the cleavage remains on the surface of the parasite as it enters the red blood cell (Blackman *et al.* 1990).

For understanding the evolution of its polymorphism, estimating frequency of allelic recombination in *msp-1* is of importance. As this locus encodes a surface antigen targeted by host immune responses, selective pressure is likely to operate to maintain polymorphism in *msp-1*. Under selective pressure, the level of polymorphism would be affected by frequency of allelic recombination. Within a locus with a high recombination rate, polymorphic sites separated by a relatively short distance segregate independently at meiosis and therefore may have different evolutionary histories (Riley *et al.* 1992).

Plasmodium species of *msp-1* exhibits extensive genetic polymorphism (Tanabe *et al.* 1987; Putaporntip *et al.* 2002) that appears to be maintained by positive natural selection in *P. falciparum* (Hughes, 1992; Escalante *et al.* 1998; Conway *et al.* 2000) and *P. vivax* (Putaporntip *et al.* 2006; Thakur *et al.* 2008). It is noteworthy that overall the *msp-1*₃₃ fragment exhibits greater genetic diversity than the *msp-1*₁₉ regardless of the species (Pacheco *et al.* 2007), however, positive natural selection was acting on the MSP-1₁₉ region, MSP-1₃₃ was neutral or under purifying selection in *P. falciparum*, while the opposite pattern was observed in *P. vivax*. The polymorphism in the *P. vivax* *msp-1*₃₃ is not evenly distributed. Indeed, in a region of 105 bp of a total of 848 bp in *msp-1*₃₃ (35 amino acids), a clear excess of non-synonymous versus synonymous substitution was observed (Manamperi, 2002; Putaporntip *et al.* 2002; Thakur *et al.* 2008) where peptides with high specific binding activity (HSBA) to reticulocytes was detected (Rodríguez *et al.* 2002; Espinosa *et al.* 2003). This pattern indicated that HSBA regions are under selective constraints to accumulate amino acid replacements; as a result, these could be a valuable target for a vaccine against *P. vivax* as has been suggested previously.

Though only two complete gene sequences of *Pvmsp-1* were characterized, analysis of specific gene regions derived from parasite isolates from Sri Lanka (Premawansa *et al.* 1993) Thailand (Putaporntip *et al.* 2000), Colombia (Mancilla *et al.* 1994) and India (Thakur *et al.* 2008) revealed new sequence types of *msp-1* polymorphic region, mostly resulting from recombination between sequences of the two original Belem and Sal I strains (Mancilla *et al.* 1994; Putaporntip *et al.* 2000). The organization and allelic recombination of the Merozoite Surface Protein-1 gene was evaluated by Putaporntip *et al.* (2002) in complete nucleotide sequences of 40 clinical isolates from various geographic areas. The mosaic organization of *Pvmsp-1* presented a highly informative pattern of intragenic recombination at the level of population sequence diversity and this study obtained evidence for heterogeneity in frequency of allelic recombination events. The chance of recombination is considerably reduced with increasing sequence divergence considering that the high number of recombination sites in conserved blocks is related to the high frequency of

recombination events. The high recombination rate of *P. vivax* has important implications for understanding the population structure of the parasite. A recent study demonstrated that *P. falciparum* has a spectrum of population structures: 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 (Anderson *et al.* 2000). Such a spectrum may also exist for *P. vivax* populations. However, the study conducted by Putaporntip and colleagues (2002) observed linkage “equilibrium” within a few kilobases of *Pvmsp-1* and a high level of heterozygosity ($h = 0.935$) of *Pvmsp-1* alleles in a *P. vivax* population from Thailand, where malaria transmission is low. Long-lasting and recurrent parasitemia often associated with relapse, a characteristic of *P. vivax* infection, may contribute to an increased rate of heterozygote formation and subsequent interallele recombination in the *Anopheles* mosquito vector. Therefore, new *Pvmsp-1* alleles could be generated by recombination events even in areas with low levels of malaria transmission (Putaporntip *et al.* 2002).

4.2. Objectives of the study

A blood stage vaccine generally aims to prevent or significantly reduce blood stage parasitemia either by reducing merozoite invasion of red cells or by targeted destruction of parasitized red cells. While progress in the development of such vaccines has been hampered by a number of factors, antigenic diversity has played a major role (Ferreira *et al.* 2004). This diversity is generated and maintained by several factors, including genetic recombination during the sexual phase of the parasite reproduction in the mosquito and positive natural selection by the host immune system (Chen *et al.* 2000; Escalante *et al.* 2004). This variation may partly explain why the acquisition of natural immunity to malaria is slow since the immune system is exposed to a constantly changing parasite population. Therefore, this study was undertaken to study the prevailing genetic diversity of the *P. vivax* merozoite surface protein-1₄₂ in Sri Lanka and to demonstrate the differential binding of predicted B and T cell epitopes to MHC alleles due to polymorphic amino acid substitutions.

The C-terminal fragment of the *P. vivax msp-1* gene was amplified using 95 isolates infected with *P. vivax* from two endemic areas for malaria (Kataragama and Anuradhapura) and a non-endemic area (Colombo) to determine the prevailing genetic diversity of this gene in Sri Lanka. Using the sequences of the amplified products the following objectives are to be studied:

1. To explore the level of diversity of the entire *Pvmmsp-1₄₂* locus as well as of the *Pvmmsp-1₃₃* and *Pvmmsp-1₁₉* fragments in the local population with the help of several measures of genetic diversity (nucleotide diversity, haplotype diversity, natural selection, recombination, gene flow).
2. To compare the population diversity indices between other geographically different countries to determine the level of diversity in geographically diverse *P. vivax* populations suggesting the importance of PvMSP-1₄₂ as a promising vaccine against *P. vivax*.
3. To identify amino acid haplotypes within the local population and to compare its polymorphism between the two reported complete sequences of PvMSP-1₄₂ (the Salvador I and the Belem strains) and to compare them with other previously reported data.
4. To construct a phylogenetic tree based on *Pvmmsp-1₄₂* nucleotide sequences obtained from the local parasite isolates as well as other reported geographically diverse isolates to examine the evolutionary relationship of its polymorphism.
5. To determine predicted B and T cell epitopes and their differential binding to class II MHC alleles as a result of non-synonymous substitutions which may evoke different host immune responses against PvMSP-1₃₃.

4.3. Results

4.3.1. *Pvmmsp-1₁₉* polymorphism

Only two synonymous mutations were observed in the 19 kDa fragment at positions 4941 and 4959 (Sal I numbering), either together or individually in 12 isolates from Colombo (N=6), Kataragama (N=5), and Anuradhapura (N=1). A single minimum recombination event (R_m) was detected between these two positions in a single isolate from Colombo. The nucleotide diversity per site (π) values for 19 kDa reported in previous studies ([Manamperi, A., 2002. Ph.D. thesis. University of Colombo, Sri Lanka]; Pacheco *et al.* 2007; Thakur *et al.* 2008) were considerably lower than that observed here due to the above two synonymous substitutions (Table 4.1). However, Tajima's D for the 19 kDa fragment had a negative value indicative of an excess of low frequency variants in the

sample (Table 4.1). The neutrality indices, obtained using the McDonald-Kreitman test with *P. cynomolgi* as the out group, showed that the 19 kDa was under purifying selection, such that polymorphism was actively suppressed by natural selection (Table 4.1.).

4.3.2. *Pvmmsp-1₄₂* / *Pvmmsp-1₃₃* polymorphism

In the 33 kDa fragment, 56 polymorphic nucleotide positions were evident from all three sites with 61 (Anuradhapura and Kataragama) and 62 (Colombo) distinct point mutations, respectively. Differences between the nucleotide diversity of non-synonymous mutations per non-synonymous site (D_n) and that of synonymous mutations per synonymous site (D_s) were indicative of positive selection acting on the 33 kDa fragment in Anuradhapura and Colombo ($D_n-D_s = 0.0152$ and 0.0141 respectively, $P < 0.05$). Values of P less than 0.05 were considered significant at the 5% level where the probability of the null hypothesis of strict-neutrality ($D_n=D_s$) was rejected in favour of the alternative hypothesis ($D_n > D_s$). In contrast, a significant departure from neutrality for Tajima's D test in the 33 kDa fragment was observed in Kataragama and Colombo (Table 4.1).

All 58 polymorphic *mmsp-1₄₂* positions were parsimony informative sites (having a minimum of two nucleotides each present at least twice), with 53 having two variants, four having three, and one having four (nucleotide position 4550). In particular, a polymorphic region of 113 bp at positions 4468-4581 having a clear excess of non-synonymous substitutions, confirmed previous reports as were overall π values for the *mmsp-1₃₃* in Table 4.1 ([Manamperi, A., 2002. Ph.D. thesis. University of Colombo, Sri Lanka]; Pacheco *et al.* 2007; Thakur *et al.* 2008). The haplotype diversity and nucleotide diversity for *Pvmmsp-1₄₂* in the entire population were 0.962 ± 0.007 S.D and 0.0231 ± 0.0005 S.D., respectively, and were also similar to those reported from India (Thakur *et al.* 2008). Comparatively elevated π values for Kataragama compared to Anuradhapura and Colombo for both the 42 and 33 kDa fragments may point at higher transmission levels in Kataragama.

Taking *P. cynomolgi* as the out group, the McDonald-Kreitman test for neutrality was applied separately to the 42 and 33 kDa fragments (Table 4.2). In all three study areas, a significant departure from neutrality on the basis of excess inter-specific non-synonymous 42 kDa polymorphisms relative to non-synonymous divergence from *P. vivax* was evident. Anuradhapura showed a particularly significant departure from neutrality ($NI=2.062$, $P < 0.001$) compared to Kataragama ($NI=2.019$) and Colombo ($NI=2.042$) indicating strong positive selection at the *Pvmmsp-1₄₂* locus. Surprisingly *PvMSP-1₃₃* had significantly less inter-specific non-synonymous polymorphism at the three sites ($P < 0.01$), providing strong evidence for purifying selection.

Of the 17 minimum recombination events (R_m) observed in *Pvmosp-I₃₃*, 11 were common to all three study sites. The linkage disequilibrium index (R^2) plotted against the nucleotide distances showed decline across the entire 1016 bp region indicating that intragenic recombination may contribute to the diversity observed in *Pvmosp-I₄₂*.

The degree of genetic differentiation between populations was estimated by the F_{ST} value. This value ranges from 0 to 1 and measures the amount of allelic diversity within and between geographically distinct populations. A value of 0 indicates all alleles are evenly distributed among populations, and 1 indicates an allele is found in only one population (Cole-Tobian and King, 2003). Comparison of the genetic differentiation within Sri Lankan *Pvmosp-I₄₂* diversity showed low F_{ST} values of -0.0205 between Anuradhapura/Kataragama, -0.0196 Anuradhapura/Colombo and -0.0177 Kataragama/Colombo. However, the F_{ST} value for the entire Sri Lankan population with other countries showed the highest F_{ST} values with Bangladesh ($F_{ST} = 0.1894$) followed by Thailand ($F_{ST} = 0.0751$), Brazil ($F_{ST} = 0.0305$) and India ($F_{ST} = 0.0039$).

Table 4.1. DNA sequence polymorphisms in *P. vivax msp-1* among Sri Lankan field isolates

	π (S.D)	Ds	Dn	Dn-Ds(S.D)	(Z-test)	Tajima's D Test
42 kDa						
Entire Population (N=95)	0.0231 (0.0005)	0.0170	0.0253	0.0083 (0.0045)	P > 0.05	2.86687 (P<0.05)
Anuradhapura (N=22)	0.0231 (0.0015)	0.0150	0.0258	0.01076 (0.0056)	P < 0.05	1.51312 (P>0.10)
Kataragama (N=36)	0.0238 (0.0009)	0.0185	0.0258	0.0073 (0.0064)	P > 0.05	2.16328 (P<0.05)
Colombo (N=37)	0.0233 (0.0008)	0.0172	0.0255	0.0082 (0.0057)	P > 0.05	1.98154 (P>0.10)
33 kDa						
Entire Population (N=95)	0.0310 (0.0007)	0.0213	0.0347	0.0134 (0.0078)	P < 0.05	2.97050 (P<0.01)
Anuradhapura (N=22)	0.0313 (0.0012)	0.0201	0.0353	0.0152 (0.0075)	P < 0.05	1.58063 (P>0.10)
Kataragama (N=36)	0.0319 (0.0011)	0.0231	0.0354	0.0122 (0.0085)	P > 0.05	2.26303 (P<0.05)
Colombo (N=37)	0.0311 (0.0011)	0.0208	0.0349	0.0141 (0.0080)	P < 0.05	2.05270 (P<0.05)
19 kDa						
Entire Population (N=95)	0.0012 (0.0003)	0.0059	0.00	-0.0058 (0.0040)	P > 0.05	-0.24254 (P>0.10)
Anuradhapura (N=22)	0.0003 (0.0003)	0.0016	0.00	-0.0016 (0.0016)	P > 0.05	-1.16240 (P>0.10)
Kataragama (N=36)	0.0013 (0.0005)	0.0063	0.00	-0.0063 (0.0046)	P > 0.05	-0.51702 (P>0.10)
Colombo (N=37)	0.0016 (0.0005)	0.0078	0.00	-0.0078 (0.0058)	P > 0.05	-0.16364 (P>0.10)

π - nucleotide diversity with standard deviation (SD)

N - number of sequences

Ds - nucleotide diversity of synonymous mutation per synonymous site, Dn nucleotide diversity of non-synonymous mutation per non-synonymous site, using the Nei and Gojobori method

Ds - Dn - the difference of Ds and Dn with their standard deviation estimated by bootstrap with 1000 pseudo replicates. Z-test was applied to test significance (Nei and Kumar, 2000)

Tajima's D - to detect departure from the neutral mode

Table 4.2 - The McDonald and Kreitman test for different regions of *msp-1*

Region		Interspecific fixed differences	Intraspecific difference		Neutrality index ¹	Fisher's Exact Test ²
			<i>P. vivax</i> (N=95)	<i>P. cynamolgi</i> (N=10)	M K test	(2- tailed)

Entire Population						
42 kDa	Synonymous	49	8	42	2.042	0.010277*
	Non-synonymous	46	43	57		
33 kDa	Synonymous	38	6	35	2.219	0.010240*
	Non-synonymous	36	43	47		
19 kDa	Synonymous	11	2	7	1.222	1.000000
	Non-synonymous	10	-	10		
Anuradhapura						
42 kDa	Synonymous	49	7	42	2.062	0.009958**
	Non-synonymous	46	42	57		
33 kDa	Synonymous	38	6	35	2.192	0.0105*
	Non-synonymous	36	42	47		
19 kDa	Synonymous	11	1	7	1.375	0.7512
	Non-synonymous	10	-	10		
Kataragama						
42 kDa	Synonymous	49	7	42	2.019	0.0105*
	Non-synonymous	46	43	57		
33 kDa	Synonymous	38	5	35	2.192	0.0105*
	Non-synonymous	36	43	47		
19 kDa	Synonymous	11	2	7	1.222	
	Non-synonymous	10	-	10		
Colombo						
42 kDa	Synonymous	49	7	42	2.042	0.010277*
	Non-synonymous	46	44	57		
33 kDa	Synonymous	38	5	35	2.219	0.010240*
	Non-synonymous	36	44	47		
19 kDa	Synonymous	11	2	7	1.222	1.000000
	Non-synonymous	10	-	10		

¹ = Neutrality index indicates the extent to which the levels of amino acid polymorphism depart from the expected in the neutral model

² = Fisher's exact test. * 0.01<P<0.05; ** 0.001<P<0.01;*** P<0.001

4.3.3. Classification and identification of haplotypes

Ninety five *Pvmosp-1₄₂* sequences in this study defined 37 nucleotide and 27 amino acid haplotypes in the 33 kDa fragment (Table 4.3) and a single 19 kDa amino acid haplotype, generally confirming previous results ([Manamperi, A., 2002. Ph.D. thesis. University of Colombo, Sri Lanka], Pacheco *et al.* 2007; Putaporntip *et al.* 2000, 2002). Interestingly, no amino acid haplotype was identical to either the Salvador I or the Belem strains across the entire 33 kDa fragment, although the H1 type differed from the Belem strain at only a single position (1432). Fourteen of the 27 amino acid haplotypes were common to all three study sites (Table 3.4). Published 202 *Pvmosp-1₄₂* sequences corresponding to Sal I amino acids 1384–1722 from diverse geographical regions were obtained from the Gene Bank to compare polymorphism with the Sri Lankan isolates.

Longacre (1995), divided the 33 kDa fragment into three segments based on its diversity: (i) N-terminal region I (RI) upstream of the hyper variable block (HVR) block with 7 variant positions, (ii) a central 38 amino acid HVR region (RII) including positions 1490-1527 with 29 variants, and (iii) region III (RIII) downstream of the HVR block and upstream of the 19 kDa fragment with 3 variant positions defined in this study (Table 3.3). All variant positions in RI and RIII and over half in the HVR (19/29) were dimorphic, where only two alternating amino acid residues observed in isolates from this and all other studies cited (Table 4.3).

RI of the present Sri Lankan cohort included 5 more variant positions compared to Manamperi's [Manamperi, A., 2002. Ph.D. thesis. University of Colombo, Sri Lanka] Sri Lankan cohort, but none of those RI haplotypes were identical to either Sal-I or Belem. Indeed all 95 isolates had S at position 1432 as for Sal-I and all but two haplotypes had K at position 1466 as for Belem, while 1454-I/L was relatively evenly distributed. Interestingly, 9 isolates defined two variant positions (1443-K>E and 1458-K>E) unique to this study (Tables 4.3 & 4.4), whereas two other variants, 1476-T>A and 1482-G>E, were frequently observed in other studies, usually paired as A-E, although A-G was occasionally seen, but never T-E (Table 3.3). Four haplotypes from other studies (H70, 76, 77, 75) defined an eighth variant position in RI (1468-L>V; Table 4.3).

In RIII, 15 of the 27 haplotypes from the present study corresponded to the single Belem-Sal-I prototype. Of the 3 variant positions, 1566-N>S was frequently observed both in Sri Lankan

isolates and in previous studies, 1586-N>T was present in only 8 Sri Lankan isolates (H25-H27) and in H66 from India, while 1595-Y>S was seen in only 6 Sri Lankan isolates (Tables 4.3 & 4.4).

Although the HVR displayed the most complex polymorphism, it was nevertheless subdivided into 7 basic types (HVR-T1 to T7) seen in 24 of the 27 haplotypes defined here (Tables 4.3 & 4.4). HVR-T1 was essentially the Belem type seen in H1-H4 that comprised 13 isolates, while HVR-T2 was represented by H19-H27 comprising 32 isolates that corresponded to the Sal-I strain (Table 4.3 & 4.4). Amino acid haplotypes H7-H11 comprising 23 isolates, H13-H14 in 11, and H17-H18 in 6 isolates defined HVR types T3, T4 and T5, respectively (Table 4.3 & 4.4). Altogether 94% of the isolates fell into one of the five HVR types, with a surprising 75% found in T1-T3. HVR-T6 comprising three isolates of H5 differed from HVR-T1 by a single variant position (1511-K>E) and HVR-T7 with a single H15 isolate differed from HVR-T4 by a single variation (1516-E>V) (Tables 4.3 & 4.4). H6, appeared to be a double recombinant between Belem (HVR-T1) and HVR-T3 (T1>T3>T1) with a single variation (1520-T>A). Nevertheless, it was observed in 3 different isolates suggesting that it represents a separate HVR type (HVR-T8). H12 of HVR-T9 represented a double recombinant between HVR-T3 and HVR-T1 (T3>T1>T3) with no other variations. The single H16 isolate appeared to be a recombinant between HVR-T2 (Sal-I) and T3, defining HVR type T10.

Markedly, of the 10 HVR types represented in the local isolates, 9 were observed in 74 global isolates, with the exception of HVR-T7, which was unique to Sri Lanka. The HVR-T1 (Belem) observed in haplotypes H28-H30 included 6 isolates from Sri Lanka [Manamperi, A., 2002. Ph.D. thesis. University of Colombo, Sri Lanka], two from India and one from South Korea, respectively, while HVR-T2 (Sal I, H62-H70) constituted the majority of global isolates (N=17). Surprisingly, HVR-T3 was identified in only three other isolates (H41-H43 from Sri Lanka, India and Thailand, respectively). HVR-T4 recurred in H51-H57 and included two isolates each from Thailand and Brazil, and one each from Bangladesh, Japan, Sumatra and Vanuatu. As, HVR-T5 was relatively rare, and included only two isolates from India (H58 and H59) (Table 4.3), HVR-T6 was seen in H31 and H32 from India (one isolate each) and H33 from Bangladesh (three isolates). Interestingly, HVR-T8, HVR-T9 and HVR-T10 recurred in five (H36-H40), four (H47-H50), and two (H60, H61) haplotypes from other studies (Table 4.3). Finally, two new HVR types T11 and T12 representing a double

recombination between HVR-T1/T3 and HVR-T6/T2, respectively were observed in haplotypes H44-H46 (4 South American isolates) and H34-H35 (8 Thai isolates). The remaining 19 haplotypes (H71-H89) defined by 21 isolates from outside Sri Lanka, were generated by intragenic recombination (Table 4.3).

Altogether, 202 sequences defined 89 amino acid haplotypes (Table 4.3), of which eight PvMSP-1₄₂ haplotypes H2, H5, H6, H7, H9, H12, H14 and H18 from this study were found in other global isolates representing HVR types T1, T3-T6 and T8-T9. While most of the haplotypes were observed in India (H5-02, H6-01, H9-02, H12-01, H14-02, H18-02), H7 included one isolate from India and two from Brazil and H2 included 3 isolates from South Korea. H28 and H63 from Sri Lanka [Manamperi, A., 2002. Ph.D. thesis. University of Colombo, Sri Lanka] was identical to the Belem and Sal I strains, respectively. Strikingly, 19 of the haplotypes described in this study are unique to the island and are reported for the first time.

Of the 47 PvMSP-1₄₂ polymorphic amino acid positions identified in this and previous studies, 28 were dimorphic, 10 were trimorphic (T/E/A-1494, K/A/T-1505, Q/E/H-1506, K/E/Q-1511, V/D/E-1516, S/T/N-1518, S/T/A-1521, N/T/A-1524, E/Q/K-1525, F/S/L-1526), and 3 were tetramorphic, (N/S/A/V-1517, D/T/A/N-1520 and I/T/M/N-1527) (Table 3.3.3). Eight previously reported dimorphic sites at positions 1468, 1512, 1573, 1574, 1597, 1601, 1612 and 1709 were not observed in this study, though two novel dimorphic sites K/E-1443 and K/E-1458 were observed (Table 4.3). Dimorphic sites at positions 1573, 1574 were observed in a single isolate from Thailand, while 1597, 1601 was observed in an Indian isolate (Table 4.3).

4.3.4. Phylogenetic tree of *Pvmsp-1*₄₂

To investigate PvMSP-1₄₂ phylogenetic relationships, we constructed a Neighbor-Joining tree based on the 27 haplotypes described here (Figure 4.1). Interestingly, the haplotypes were clustered into eight groups as shown by the 8 different HVR types (HVR-T1-T8) at PvMSP-1₃₃. Haplotype H1 through H4 and H19 through H27 closely related to the Belem and Sal I strains respectively, and represented HVR types T1 and T2. Haplotype H7 through H11, H13 through H14, H17 through H18 and H6, H12, H16 defined six separate groups that

represented the observed HVR types T3-T5 and T8-10, respectively. Though, haplotypes H5 and H15 represented unique HVR types they were clustered with haplotypes of HVR-T1 and T4, respectively due to the variation of a single amino acid each.

A phylogenetic tree constructed using all published 202 *Pvmsp-1₄₂* nucleotide sequences defined 12 distinctive groups (Figure 4.2). As indicated above, the Sri Lankan isolates were clustered in 5 of the 12 groups, with a majority belonging to group 12. Groups 6 and 7 comprised sequences only from this study. Group 12 (Sal I) also included isolates from Vietnam, Thailand, India and Brazil and one isolate each from Bangladesh, Japan and Sumatra (Pacheco *et al.* 2007; Putaporntip *et al.* 2002), where as the Belem isolate was not clustered in a single group. Groups 2, 4 and 11 included Thai isolates, while groups 5 and 10 were exclusively from India. Groups 1 and 8 comprised isolates from Bangladesh and Brazil. Two isolates from Colombo were clustered in group 9 with four Thai, two Indian and one Brazilian isolate. Group 3 consisted of a sub group of three Sri Lankan isolates from this study (one from each study area) and three isolates from India.

Group 12 comprised six sub-groups, where only two included isolates from our study, one with 9 Sri Lankan isolates closely related to an isolate from Bangladesh, and another with 6 Sri Lankan isolates related to 4 from India. The other sub groups contained isolates related to Brazil, Thailand and Vanuatu (Figure 4.2).

4.3.5. Prediction of B and T-cell epitopes in PvMSP-1₃₃ and PvMSP-1₁₉

Three B cell epitopes were predicted at amino acid positions 1393, 1542 and 1587 of the 33 kDa fragment in all 27 haplotypes. While H1 lacked a B cell epitope due to amino acid substitution at 1432, the other haplotypes included supplementary predicted B cell epitopes due to amino acid substitutions at several positions. The 19 kDa fragment encompassed three predicted B cell epitopes at amino acid positions 1615, 1657 and 1678.

Predicted T cell epitopes on MSP-1₃₃ shared by all 27 haplotypes were clustered on either side of the hypervariable region and included 1 for HLA-DRB1*0101, 3 for HLA-DRB1*0401, 2 for HLA-DRB1*0701 and 4 for HLA-DRB1*1101 (Figure 4.3). Additionally common T cell epitopes were predicted for H1-H6, as well as a novel H3 epitope.

Polymorphism in the hypervariable regions of H7-H27 generated diverse predicted T cell epitopes for all of the HLA alleles, except HLA-DRB1*0701. While two MSP-1₁₉ T cell epitopes were predicted for HLA-DRB1*0101 and one for both HLA-DRB1*0401 and 1101, the epitope LLNVQTQLL predicted for HLA-DRB*0701 was positioned at the end of MSP-1₃₃ and beginning of MSP-1₁₉ (Figure 3.3).

Table 4.3. Amino acid polymorphism in the *P. vivax* MSP-1₃₃ among geographically diverse malaria endemic areas

[illegible]

Amino acid haplotypes

A

N

Strain	Position	Sequence	Group
H1	6	. . L . K . . . T A N E . V A . A E . D K I . . I . S D S T K T T E T Q S M	HVR-T1
H2	2	. . L . K . A E T A N E . V A . A E . D K I . . I . S D S T K T T E T Q S M	
H3	1	. . L . K . . . T A N E . V A . A E . D K I . . I . S D S T K T T E T Q S M	
H4	4	 K . A E T A N E . V A . A E . D K I . . I . S D S T K T T E T Q S M	
H5	3	 K . . . T A N E . V A . A E . D K I E . I . S D S T K T T E T Q S M	HVR-T6
H6	3	 K . A E T A N E . V A . A E . D K I . . I E . E A N K A A E T Q S M	HVR-T8
H7	11	. . L . K . A E T A N A Q . . N T . N I E . E A N K T A E A K . T	HVR-T3
H8	1	. E L . K . A E T A N A Q . . N T . N I E . E A N K T A E A K . T	
H9	8	 K . A E T A N A Q . . N T . N I E . E A N K T A E A K . T S	
H10	2	. E . E K . A E T A N A Q . . N T . N I E . E A N K T A E A K . T S	
H11	1	 K . A E T A N A Q . . N T . N I E . E A N K T A E A K . T	
H12	2	 A E T A N A Q . . N T . N I E . D S T K T T E A K . T	HVR-T9
H13	9	 A E T H . . K . Q . . . S E V . . N . E T Q L N S	HVR-T4
H14	2	. . L . K T H . . K . Q . . . S E V . . N . E T Q L N	
H15	1	. E L E K T H . . K . Q . . . S . V . . N . E T Q L N	HVR-T7

Note: Amino acid variant positions 1432 – 1709 corresponded to the two reference strains of PvMSP-1₃₃ Belem* (Accession no. AF435594; Putaporntip *et al.* 2002) and Salvador I[#] (Accession no. M75674; Gibson *et al.* 1992). Positions of amino acid substitutions are specified vertically above with reference to the Salvador I strain. Pink and light blue correspond respectively to Belem and Sal I specific residues; yellow residues are identical between Belem and Sal I; green residues differ from both Belem and Sal I. HVR-T1 to T12 represents the PvMSP-1₃₃ hypervariable region sequence types of (A) Amino acid haplotypes from the current study, (B) Amino acid haplotypes of global isolates, and (C) Amino acid haplotypes manifested by intragenic recombination.

Table 4.4. The numbers of isolates present in each of the PvMSP-1₄₂ amino acid haplotypes representing the three study areas of Sri Lanka

Amino Acid Haplotypes	Study Area		
	Anuradhapura	Kataragama	Colombo
	N = number of isolates		
H1	√ (3)	√ (3)	
H2	√ (1)		√ (1)
H3		√ (1)	
H4		√ (1)	√ (3)
H5			√ (3)
H6	√ (1)	√ (1)	√ (1)
H7	√ (1)	√ (7)	√ (3)
H8		√ (1)	
H9	√ (3)	√ (2)	√ (3)
H10		√ (1)	√ (1)
H11			√ (1)
H12			√ (2)
H13	√ (2)	√ (5)	√ (2)
H14	√ (1)		√ (1)
H15	√ (1)		
H16			√ (1)
H17		√ (1)	
H18	√ (2)	√ (1)	√ (2)
H19	√ (1)		√ (1)
H20	√ (1)	√ (4)	√ (4)
H21		√ (1)	√ (1)
H22	√ (4)	√ (1)	√ (4)
H23			√ (1)
H24		√ (1)	
H25		√ (1)	
H26	√ (1)		
H27		√ (4)	√ (2)

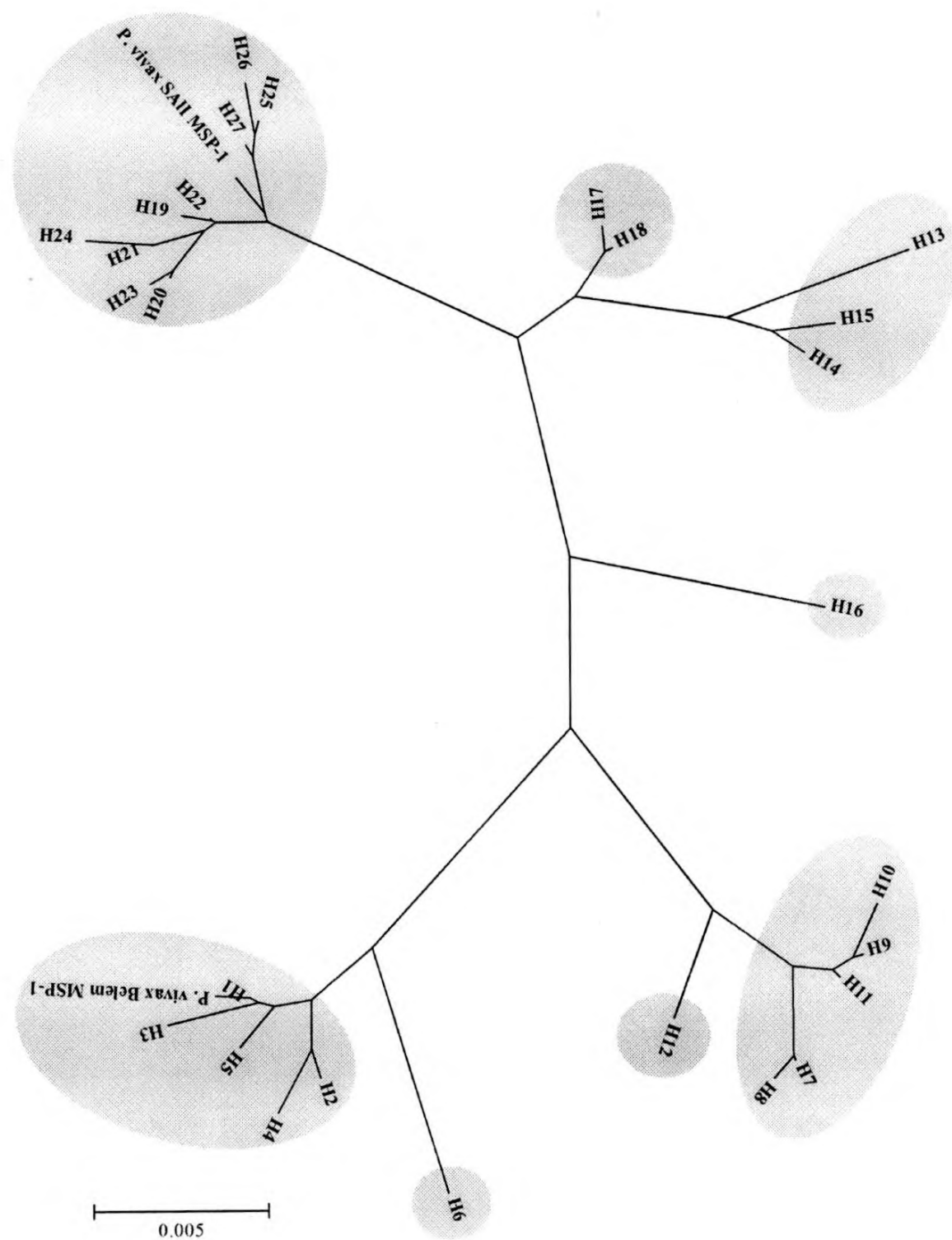


Figure 4.1. Neighbour-Joining tree of PvMSP-1₄₂ amino acid sequence of 27 haplotypes observed among 95 Sri Lankan isolates

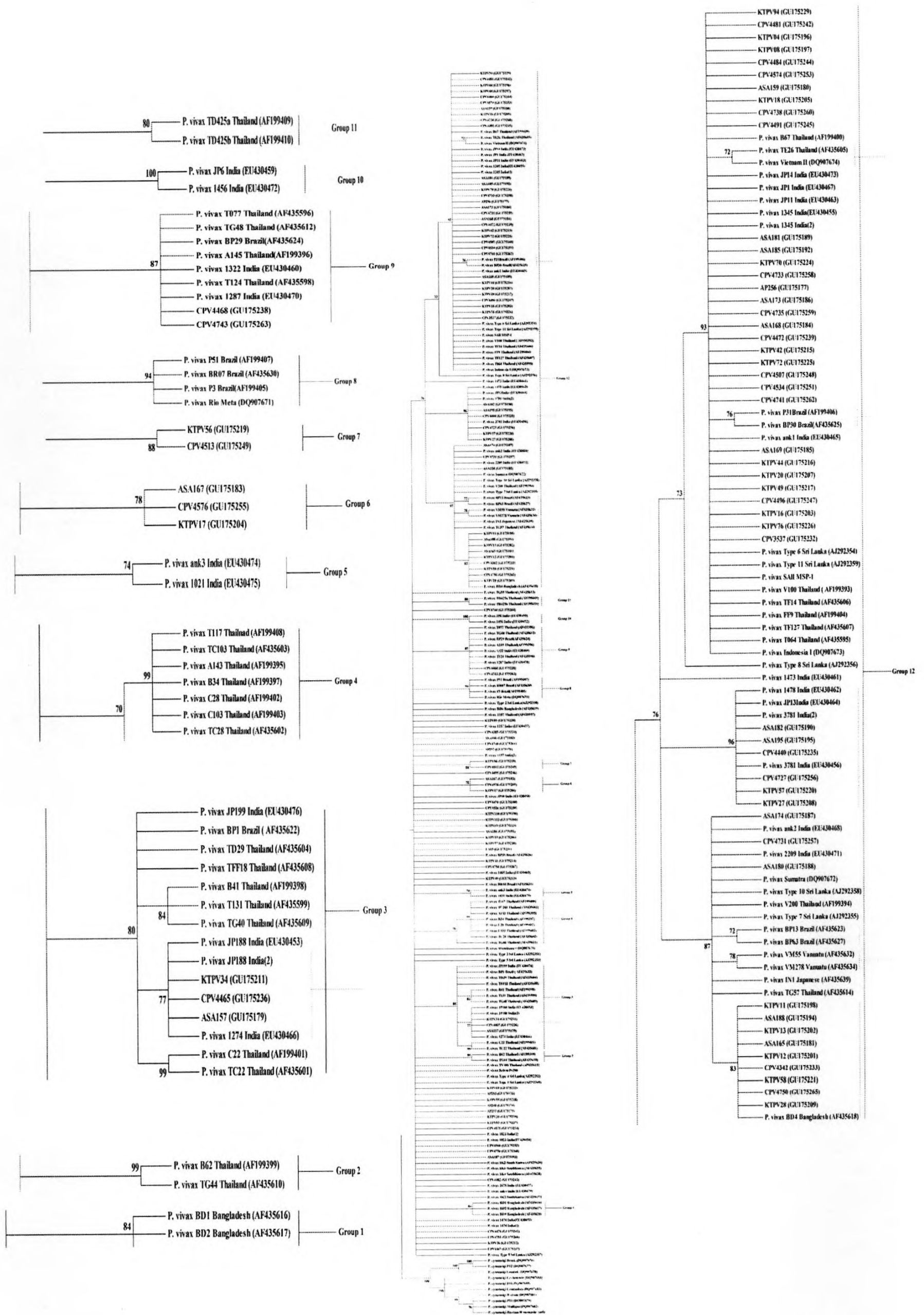


Figure 4.1. Neighbour-Joining tree of the *PvmSP-1* using Kimura 2 parameter

Isolates of Sri Lanka and other geographically different countries are included. The numbers on the node of the tree are percent bootstrap values based on 1000 pseudo-replications, only bootstrap values above 70% are displayed on the tree.

DRB1_0101:MYKTIKKQLENHVNAFNTNITDMLDSRLKKRNYFLEVLNSDLNPFKYSPSGEYIHKDPYKLLDLEKKKKLLGSYKYIGASIDKDLATANDGVITYYNKM
GELYKTHLTAVNEEVKKVEADIKAEDDKIKKIGSDSTKTEKTOSMAKKAELEKYL PFLNSLQKEYESLVSKVNTYTDNLKKVINNCQLEKKEAEITVKKLQDYNK
MDEKLEEYKKSEKKNEVKSSGLEKLMKSKLIKENESKEILSQLLNVQTQLLTMSSEHTCIDTNVPDAAACYRYLDGTEEWRCCLTFKEEGGKCPASNVTCDDN
GGCAPEAECKMTDSNKIVCKCTKEGSEPL

DRB1_0401:MYKTIKKQLENHVNAFNTNITDMLDSRLKKRNYFLEVLNSDLNPFKYSPSGEYIHKDPYKLLDLEKKKKLLGSYKYIGASIDKDLATANDGVITYYNKM
GELYKTHLTAVNEEVKKVEADIKAEDDKIKKIGSDSTKTEKTOSMAKKAELEKYL PFLNSLQKEYESLVSKVNTYTDNLKKVINNCQLEKKEAEITVKKLQDYNK
MDEKLEEYKKSEKKNEVKSSGLEKLMKSKLIKENESKEILSQLLNVQTQLLTMSSEHTCIDTNVPDAAACYRYLDGTEEWRCCLTFKEEGGKCPASNVTCDDN
GGCAPEAECKMTDSNKIVCKCTKEGSEPL

DRB1_0701:MYKTIKKQLENHVNAFNTNITDMLDSRLKKRNYFLEVLNSDLNPFKYSPSGEYIHKDPYKLLDLEKKKKLLGSYKYIGASIDKDLATANDGVITYYNKM
GELYKTHLTAVNEEVKKVEADIKAEDDKIKKIGSDSTKTEKTOSMAKKAELEKYL PFLNSLQKEYESLVSKVNTYTDNLKKVINNCQLEKKEAEITVKKLQDYNK
MDEKLEEYKKSEKKNEVKSSGLEKLMKSKLIKENESKEILSQLLNVQTQLLTMSSEHTCIDTNVPDAAACYRYLDGTEEWRCCLTFKEEGGKCPASNVTCDDN
GGCAPEAECKMTDSNKIVCKCTKEGSEPL

DRB1_1101:MYKTIKKQLENHVNAFNTNITDMLDSRLKKRNYFLEVLNSDLNPFKYSPSGEYIHKDPYKLLDLEKKKKLLGSYKYIGASIDKDLATANDGVITYYNKM
GELYKTHLTAVNEEVKKVEADIKAEDDKIKKIGSDSTKTEKTOSMAKKAELEKYL PFLNSLQKEYESLVSKVNTYTDNLKKVINNCQLEKKEAEITVKKLQDYNK
MDEKLEEYKKSEKKNEVKSSGLEKLMKSKLIKENESKEILSQLLNVQTQLLTMSSEHTCIDTNVPDAAACYRYLDGTEEWRCCLTFKEEGGKCPASNVTCDDN
GGCAPEAECKMTDSNKIVCKCTKEGSEPL

Figure 4.3. Predicted T cell epitopes of the PvMSP-1₄₂ Belem strain obtained for the HLA-DRB1*0101, HLA-DRB1*0401, HLA-DRB1*0701 and HLA-DRB1*1101 alleles

Predicted T cell epitopes to each MHC allele is highlighted in blue with the first position highlighted in red. The hypervariable region of PvMSP-1₄₂ is underlined and the polymorphic epitopes are circled.

4.3.6. Strain-specific immunity to PvMSP-1₄₂

Of the 95 amino acid sequences of PvMSP-1₄₂ clinical isolates, the homologous antibody responses against the recombinant PvMSP-1₄₂ representing the Belem strain and the PvMSP-1₁₉ were available for 72. The amino acid sequence homology of the 72 clinical isolates to the entire 42 kDa region of the Belem strain ranged from 91.4-99.7% (Figure 4.4). With increased amino acid haplotype numbering from H1-H27 (where the homology percentage to the Belem strain sequentially declined while it was vice versa to that of the Sal-1 strain), two clear groups of antibody responders were observed. In the group with amino acid homology ranging from 99.7-96.7% (included 1-11 amino acid substitutions out of a total of 39 spanning the entire 42 kDa region an anti-PvMSP-1₄₂ antibody prevalence of a 100% being evident in individuals representing amino acid haplotypes H2-H6 with a 99.4-96.7% homology to Belem PvMSP-1₄₂ strain may suggest strain-specific immune responses. Then again, the amino acid sequences of the homology group 93.8-91.4%, where majority of individuals (68%) assembled, those with 98.5% and 99.1% homology to the Salvador I strain, and those with 93.8- 91.7% homology to the Belem strain both recording a 100% anti-PvMSP-1₄₂ antibody prevalence, may imply strain-transcending (cross reactive) immune responses.

Further, anti-PvMSP-1₄₂ antibody prevalence varied to haplotypes with identical homology percentage (but with dissimilar amino acid sequences) to the Belem PvMSP-1₄₂; for e.g. antibody prevalence ranged from 80-100% to haplotypes H9, H13 and H27 with amino acid homolog of 92.3%, 50-100% to haplotypes H10, H19, H20, H21 and H26, with amino acid homolog of 91.7% and 0-60% to haplotypes H22 and H25 with amino acid homology of 92% to the Belem strain. Serum of haplotypes H8, H24 and H25 lacked antibodies against both recombinant PvMSP-1₄₂ and 19 proteins, while isolates of haplotypes H1, H7, H9, H19, H20, and H22 recorded both responders (ranging from 50-88.9%) and non-responders (11.1-40%) against recombinant PvMSP-1₄₂, where the highest responding prevalence was recorded against both haplotypes H7 and H20. That, malaria naïve individuals (individuals who were exposed to the infection for the

first time) from Colombo demonstrated a 100% anti-PvMSP-1₄₂ antibody prevalence against parasite haplotypes H6, H7, H9, H17 and H27 prove the immunogenicity of PvMSP-1₄₂.

Though, all 72 amino acid sequences of clinical isolates were identical to the PvMSP-1₁₉ recombinant construct, the anti-PvMSP-1₁₉ antibody prevalence was restricted to 64.7%. Moreover, of the 35.3% non-responders, 67% were residents of malaria endemic Kataragama, while 79% were exposed to the infection 3 times or more and 37.5% lacked antibodies against both PvMSP-1₄₂ and PvMSP-1₁₉.

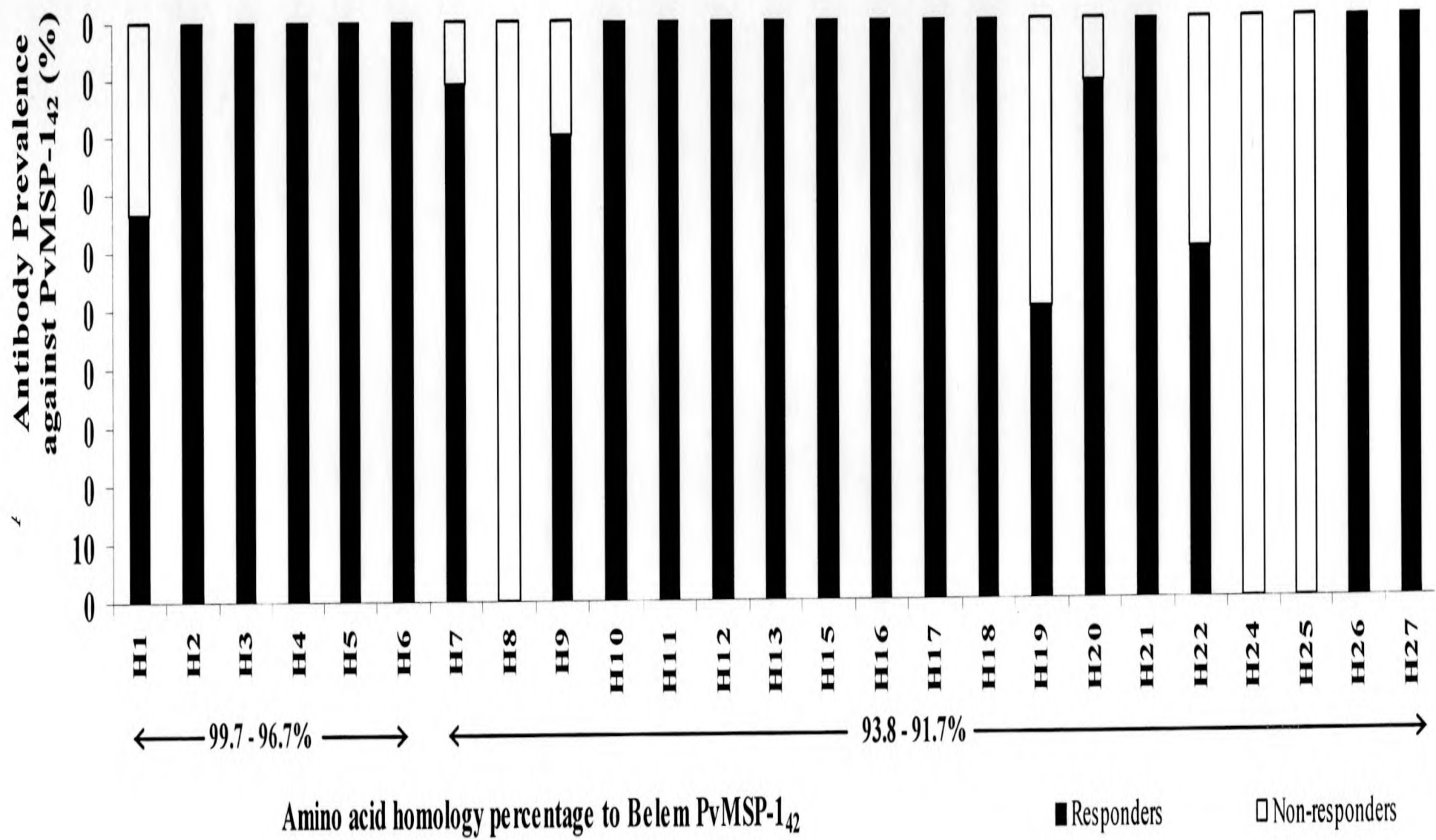


Figure 4.4. Distribution of the amino acid homology percentage to the PvMSP-1₄₂ Belem strain and the antibody prevalence against PvMSP-1₄₂ recombinant protein (Belem strain) of the manifested 27 amino acid haplotypes from Sri Lanka

4.4. Discussion

The merozoite surface protein-1₄₂ (MSP-1₄₂) is considered a leading vaccine candidate for both *P. vivax* and *P. falciparum* malaria, though extensive genetic polymorphism is a major impediment for vaccine development. Many factors may contribute to genetic diversity in malaria populations; mutation, recombination, gene flow between different populations and natural selection (Cole-Tobian and King, 2003). This study was undertaken to investigate the existing polymorphism in the *Pvmsp-1₄₂* gene in *P. vivax* clinical isolates of Sri Lanka and to identify the mechanisms used to maintain polymorphism, presumably to influence immunological responses to the parasites advantage.

In Sri Lanka *Pvmsp-1₃₃* exhibited greater genetic diversity due to 62 point mutations and the 38 residue highly polymorphic amino acid region defined as the hypervariable region (HVR), confirming previous reports (Pacheco *et al.* 2007; Thakur *et al.* 2008). Though, none of the 27 PvMSP-1₄₂ amino acid haplotypes were identical to either the Salvador I or the Belem reference strains, most resembled the Sal I phenotype at the HVR region that was reflected in the phylogenetic tree.

Four of the nine HVR types from Sri Lankan isolates recorded by [Manamperi, A., 2002. Ph.D. thesis. University of Colombo, Sri Lanka] were observed in the current study. Nine of the 10 HVR types described here were also found in global isolates, and are reported for the first time in this study, where the HVR types T8-T10 resulted due to double interallelic recombination. Interestingly, the phylogenetic tree showed that isolates of HVR-T1 and T2 types are essentially of Asian origin, except for 2 Brazilian isolates of the T2 type, and also that the novel HVR types T11 and T12 were clustered into two clear groups.

Recombination sites were not uniformly distributed across *Pvmsp-1₄₂*, with most recombination events (Rm) occurring at *Pvmsp-1₃₃*, which corroborates previous findings (Putaporntip *et al.* 2002). These accounts for HVR types T3-T7 and the 19 haplotypes (H71-84) generated solely due to intragenic recombination and further confirms that the

extensive polymorphism present at PvMSP-1₃₃ is maintained by both interallelic and intragenic recombination. A recent study demonstrated that *P. falciparum* has a spectrum of population structures: 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 (Anderson *et al.* 2000). However, linkage disequilibrium of *Pvmsp-1*₄₂ in Sri Lankan isolates showed a significant decline with increasing nucleotide distance and a high level of haplotype diversity ($h = 0.962$). These observations indicate that new PvMSP-1₄₂ alleles could be generated by recombination events even in areas with low transmission of malaria.

The fact that certain haplotypes common to Colombo and the two endemic areas may be due to; (i) Colombo patients contracting the disease during visits to endemic areas due to occupational commitments (Fonseka and Mendis, 1987; Briët *et al.* 2003), where the origin of these infections was traced to ten administrative districts that represented both the intermediate and the dry zone of the island, including the two endemic areas of this study, and (ii) the low population structure denoted by low F_{ST} values among the three study areas. The phylogenetic tree further confirmed the low genetic differentiation due to migration between Sri Lanka and neighbouring India with a majority of common amino acid haplotypes, as well as the marked genetic differentiation between Sri Lanka and Bangladesh shown by the separate clustering of 3 of the 5 Bangladesh isolates. Overall, the phylogenetic tree showed no evidence of geographic clustering of alleles, but rather the interchange of *Plasmodium* strains due to migration of individuals and/or malaria transmitting vectors.

In Sri Lankan isolates, PvMSP-1₃₃ was subjected to positive selection, with frequent occurrences of non-synonymous substitutions relative to synonymous ones, presumably to avoid host immune pressure. These results are consistent with previous studies that used a global sample of *P. vivax* isolates (Pacheco *et al.* 2007; Thakur *et al.* 2008). Consequently, these polymorphisms are actively maintained in *P. vivax* populations, and host immune responses likely play a role in maintaining the polymorphism of *P. vivax* MSP-1 alleles. Two mechanisms that enable PvMSP-1₃₃ positive selection in the local

isolates are; (i) the presence of three of the five positive selection sites essential for immune evasion by the parasite, at amino acid positions 1490 (D/T), 1491 (G/A) and 1494 (T/A/E) in the 33 kDa fragment (Tanabe *et al.* 2007), and (ii) clusters of non-synonymous substitutions occurring in the predicted T cell epitopes in all 27 PvMSP-1₃₃ amino acid haplotypes mediating differential binding of these polymorphic peptides to multiple class II MHC alleles.

Extensive genetic diversity at the *Pvmsp-1*₃₃ locus in Sri Lanka where low transmission and unstable malaria conditions persist highlights both interallelic and intragenic recombination as critical factors for the origin of new PvMSP-1₄₂ amino acid haplotypes in local *P. vivax* populations. Moreover, positive selection for polymorphism acting on PvMSP-1₃₃ in response to host immune pressure, creates new B-cell epitopes requiring new antibody responses and provides for broad B and T cell recognition, which may contribute to the “immature” natural antibody response demonstrated against PvMSP-1₄₂ in Sri Lanka (Wickramarachchi *et al.* 2007).

A single amino acid variant at position 1709 (K/E) in PvMSP-1₁₉ observed in previous studies (Pasay *et al.* 1995; Soares *et al.* 1999; [Manamperi, A., 2002. Ph.D. thesis. University of Colombo, Sri Lanka]; Putaporntip *et al.* 2000, 2002), was not observed in any of the 95 Sri Lankan isolates described here. Conversely, with the exception of a single recombination event, PvMSP-1₁₉ at the amino acid level was highly conserved in all 95 Sri Lankan isolates, with confirmed lack of T cell epitope variations. Thus, PvMSP-1₁₉ is clearly under purifying selection (Pacheco *et al.* 2007; Thakur *et al.* 2008), and the protective nature of the natural antibody response directed against the 19 kDa fragment previously characterized by the marked switch of anti-PvMSP-1₁₉ IgM to functionally important cytophilic IgG1 and IgG3 antibodies with increasing exposure to malaria in the endemic areas of Sri Lanka (Wickramarachchi *et al.* 2007), emphasize the potential of an experimental vaccine based on PvMSP-1₁₉ to be effective against *P. vivax* infections in Sri Lanka.

At PvMSP-1₄₂, those haplotypes that were more homologous to PvMSP-1₄₂ Belem strain (H2-H6 with a 99.4-96.7% homology) demonstrated a strain-specific immune response against PvMSP-1₄₂. Cortés and co-workers (2005) documented that a small subset of plasma samples from PNG showed dramatic differences in the recognition of different forms of the complete AMA-1 ectodomain, indicating that these samples predominantly contain antibodies against polymorphic epitopes that are present in some of the alternative forms but not others. However, it might be the consequence of random repeated exposure to parasites with similar forms of some polymorphic epitopes or, alternatively, it might reflect genetic characteristics of the human host that determine the capacity to mount an antibody response against particular epitopes (Cortés *et al.* 2005). Similar observations were seen from other malaria vaccine candidates as well. A study showed significant levels of cross reactive antibodies and allele specific antibodies generated against different PfMSP-1₁₉ allelic forms in a *P. falciparum* infected natural human population of India. Presence of PfMSP-1₁₉ allele specific antibodies observed in this population suggested that for a better protection against all strains of *P. falciparum*, it will be appropriate to use a vaccine containing all possible sequence variants (Mamillapalli *et al.* 2007).

However, majority of *P. vivax* parasite haplotypes of local field isolates that were less homologous to the Belem 42 kDa fragment were 100% responsive against the recombinant PvMSP-1₄₂ protein resembling the Belem strain, demonstrating that genetically disparate recombinant protein serologically cross-reacted with a majority of the Sri Lankan isolates. The tertiary structure predicted for the hypervariable block of PvMSP-1₄₂ suggests that this domain may form a highly exposed immunogenic loop comprising many cross-reactive B cell epitopes among different haplotypes (Manamperi, 2002). Franks and colleagues (2003) reported that the cross-reactive epitopes are immunodominant, such that the majority of antibodies binding to epitopes within the polymorphic domains recognise cross-reactive rather than allele-specific epitopes. However, the response characterized by multiple cross-reactive epitopes and low-affinity antibodies was suggestive of an immature immune response (Wickramarachchi *et al.* 2007). Differential binding of individual serum samples against cross-reactive proteins

indicated that the major effect of sequence variation may be modifying the affinity or avidity of antigen-antibody interactions rather than leading to complete abolition of antibody binding sites (Franks *et al.* 2003). Moreover, repeated infection with genetically distinct parasites leads to boosting of the existing antibody response rather than generation of novel specificities (Taylor *et al.* 1996), which is termed “original antigenic sin” (Riley *et al.* 1996). Therefore, it may be possible that malaria patients previously exposed to different parasite strains in the local isolate pool had developed antibodies compatible with the recombinant protein with a disparate amino acid homology to these isolates.

Individuals harbouring clinical isolates of haplotypes H8, H24 and H25 of PvMSP-1₄₂ that lacked antibody responses against both 42, 19 kDa fragments and PvAMA-1 (Wickramarachchi, 2005), together with those included in the 35.3% that did not respond to the recombinant PvMSP-1₁₉ identical to the amino acid sequence of all 72 Sri Lankan isolates, may reflect other genetic factors of the host, such as HLA polymorphism of individuals and also “immunological tolerance”. A study done by Johnson *et al.* (2004) demonstrated that though no association was found between a single HLA allele and levels of antibodies to MSA-2, MSP-1-190L, or any of the MSP-1₁₉ variants, an association was observed for AMA-1, in combination with the association between HLA class II alleles and levels of antibodies to rhoptry-associated protein 1 (RAP-1) and RAP-2. Interestingly, 67% of the non-responding individuals were observed from Kataragama, a typical *P. vivax* endemic area in Sri Lanka (Gunewardena *et al.* 1994), with an annual parasite incidence of 10 –20 per 1,000 population during the study period (Briët *et al.* 2003). Therefore, immunosuppression/ immunodepression (Pachner, 1997) may be another plausible explanation for the non-responsiveness to the 19 kDa. A majority of serum samples (62.5%) lacking antibodies to recombinant PvMSP-1₁₉, manifested antibodies against the entire recombinant PvMSP-1₄₂ protein, indicative of a probable antibody response solely against the 33 kDa region.

A strain-transcending immune response was clearly prevalent against the PvMSP-1₄₂ protein, in that PvMSP-1₄₂ strains differing in amino acid homology/sequence recognised

the recombinant PvMSP-1₄₂ protein resembling the native Belem construct. Though, anti-PvMSP-1₁₉ antibody prevalence precludes strain-specific immune response against the 19 kDa fragment, this region clearly elicit a protective mature IgG immune response with increasing exposure (Wickramarachchi *et al.* 2007). The PvMSP-1₄₂ conversely elicited an immature immune response, and the immunological “cross talk” between epitopes of PvMSP-1₄₂ and PvMSP-1₁₉ may interfere with the development of a mature IgG dominated protective antibody response, which in turn may complicate vaccine development (Wickramarachchi *et al.* 2007).

Chapter 5. Genetic diversity of domain II of *Plasmodium vivax* Apical Membrane Antigen-1 in Sri Lanka

5.1. Introduction

Merozoite antigens are under considerable investigation as potential vaccine candidates despite the extensive sequence variation within and between populations, and the evidence that antibodies raised to one variant is cross-reactive with other variants from the same species (Franks *et al.* 2003; Remarque *et al.* 2008). One such antigen that showed promise as a vaccine candidate is the Apical Membrane Antigen-1 (AMA-1). Initial studies in *Plasmodium* parasites showed that AMA-1 was a merozoite membrane protein; however, structural and functional data have identified AMA-1 in sporozoites as well (Silvie *et al.* 2004). AMA-1 is stored in the microneme organelles immediately after synthesis and is transported to the parasite surface just prior to, or during, host-cell invasion (Remarque *et al.* 2007).

Apical Membrane Antigen-1 is a structurally conserved type I integral membrane protein varying between 556 to 563 amino acids (aa) in most *Plasmodium* species (It is 622 amino acids long in *Plasmodium falciparum* and the closely related chimpanzee malaria *Plasmodium reichenowi*) (Chesne-Seck *et al.* 2005). The *ama-1* gene comprises an N-terminal ectoplasmic region, a single transmembrane region and a small cytoplasmic domain. Sixteen invariant Cysteine (Cys) residues are encoded in the ectoplasmic region of all characterized *Plasmodium* AMA-1. The disulfide binding pattern prompted the suggestion that the mature ectodomain folds into an N-terminal pro-sequence and to three domains (DI, DII and DIII) (Hodder *et al.* 1996; Pizarro *et al.* 2005). Based on the crystal structure discussed by Pizarro and group, (2005), the domains of PvAMA-1 were defined as follows: domain I, which extends from residues 43 to 248, domain II from 249 to 385, and domain III from 386 to 487. Residues are numbered from the first residue of the signal sequence; however, following alignment with the PfAMA-1 sequence, the prosequence of PvAMA-1 was assumed to terminate at residue 42 (Howell *et al.* 2001). The core of domains I and II is based on the PAN folding motif (Plasminogen, Apple, Nematode), a protein fold describing domains commonly associated with carbohydrate or protein-receptor binding functions (Tordai *et al.* 1999). These proteins are identified in plasminogen-related proteins and in

several nematode proteins. In the crystal structure of PvAMA-1, a 40-residue segment in domain II, comprising residues 295–334, had no corresponding electron density in the fourier maps and thus could not be traced. This region is therefore disordered because of conformational heterogeneity or mobility and is referred to as the domain II loop (Pizarro *et al.* 2005). Further investigations evidenced that the base of the domain II loop includes the epitope of the anti-PfAMA-1 invasion-inhibitory monoclonal antibody, 4G2. The location of the 4G2 epitope and currently known structure–function correlations of PAN domains led to the speculation that AMA-1 may have a receptor-binding role involving domain II. The loop is strictly conserved in residue length within the different *Plasmodium* orthologues but has lower sequence identity (21%) compared to the complete ectoplasmic region (39%). Nonetheless, no polymorphic sites were detected to date in the domain II loop in *P. falciparum*, (Chesne-Seck *et al.* 2005) as well as *P. vivax* (Thakur *et al.* 2008) suggesting that functional constraints may operate to prevent antigenic variation in this surface exposed segment.

An earlier study amplifying the *P. falciparum ama-1* ectodomain gene with three overlapping templates revealed that evidence of selection was strongest for domains I and III (Polley and Conway, 2001). *P. falciparum ama-1* from various countries including India showed a higher rate of non-synonymous mutations and level of diversifying selection at domain I (Cortes *et al.* 2003; Garg *et al.* 2007). Studies conducted to explore the geographical sequence variations in *P. vivax ama-1* are limited, and include those conducted on domain I (Cheng and Saul, 1994; Figtree *et al.* 2000; Han *et al.* 2002; Chung *et al.* 2003; Greenberg *et al.* 2007 and 2008) and a few on the other two domains (domain II and domain III) in a limited number of isolates that revealed few new haplotypes (Gunasekera *et al.* 2007; Rajesh *et al.* 2007; Ord *et al.* 2008).

Greenberg *et al.* (2008) conducted a study to characterize the world wide genetic diversity of domain I of *Pvama-1* to test the hypothesis that the pattern of genetic diversity at the *Pvama-1* locus reflects population history. In particular, the reduction of the effective population size of *P. vivax* in the New World, as *P. vivax* is believed to have originated in Southeast Asia. Theoretically, it is expected that if the mutation rate and selection coefficient are constant the effective population size will be the major factor determining gene diversity even at a locus under balancing selection (Reviewed in Greenberg *et al.* 2008). These results showed that genetic diversity in *P. vivax ama-1* reflects population history, with population substructure

indicative of long-established Old World populations, whereas Brazilian populations showed evidence of loss of diversity and recent population expansion (Greenberg *et al.* 2008).

Ord and colleagues (2008) presented a full length sequence analysis of *Pvama-1* and *Pfama-1* from cross sectional surveys of malaria parasites co-circulating among the human population of Venezuela, Amazon. They used these homologous sequence data sets to directly compare genetic structure between the two geographically defined parasite populations sharing the same human host, to contrast selective forces acting upon the two genes, and to examine any evidence of direct interplay between the two species in this setting of meso-endemic, sympatric transmission. The two distinct population structures observed were unlikely to result from different selective forces acting upon the two species, which share both human and mosquito hosts in this setting. Rather, the highly structured *P. falciparum* population appeared to be the result of a population bottleneck, while the much less structured *P. vivax* population was likely to be derived from an ancient pool of diversity, as reflected in a larger estimate of effective population size for this species.

Predominant prevalence of natural antibodies to *P. vivax* AMA-1 was demonstrated in populations with lifelong exposure to malaria in Brazil and in Sri Lanka (Rodrigues *et al.* 2005; Wickramarachchi *et al.* 2006). However, it was imperative to reason out the non-responsiveness of approximately 45% of endemic residents against the recombinant Pv66/AMA-1 in Sri Lanka in the latter study. Previous immuno-epidemiological studies had indicated a lack of 100% antibody prevalence to malaria antigens even among highly exposed populations to malaria, which probably reflects genetic control of host antibody responses (Egan *et al.* 1995; Shi *et al.* 1996; Taylor *et al.* 1998; Wang *et al.* 2001). The possible mechanism of genetic control was explained by introducing several mechanisms and hypothesis of which, the influence of human MHC alleles on immune responsiveness to epitopes of antigens play a major role. Although evidence on the association between MHC genotype and susceptibility to severe malaria is available, very few associations have been observed for specific antibody responses to defined malarial antigens (reviewed in Riley *et al.* 1996).

The immune responses induced may be effective in controlling growth of parasites expressing the same form of the antigen used for the immunization, but would affect the growth of parasites expressing alternative forms to a much lesser extent (Crewther *et al.*

1996; Renia *et al.* 1997; Hodder *et al.* 2001). Experiments with rodents documented that blood stage immunity induced against a single strain of parasite, lacked protection of the animal against re-infection with a genetically different strain of parasite, nor did it fully protect against the clinical symptoms of the disease (Cheesman *et al.*, 2006). Unless the antigen types previously experienced were the same, each new infection will be poorly protected until all antigen types in the prevailing parasite population have been experienced. This has become the most commonly cited hypothesis to account for the length of time taken to achieve effective protective immunity against malaria in humans exposed to malaria transmission in areas of endemicity (Cheesman *et al.* 2006). Using different allelic forms of the entire AMA-1 ectodomain and some individual sub domains, Cortés and associates (2005) characterised the antibody responses against AMA-1 in individuals from Papua New Guinea exposed to malaria which illustrated that, a majority of individuals had very high levels of antibodies against AMA-1, where the prevalence and titer increased with age. Although antibodies against conserved regions of the molecule were predominant in a majority of the individuals, most plasma samples also contained antibodies directed against polymorphic regions of the antigen.

5.2. Research design

Studies on population diversity of malaria parasites have practical significance on the development for strategies of disease control, including vaccine development (Cui *et al.* 2003). The primary factors affecting genetic diversity at such loci are natural selection (Escalante *et al.* 2004), recombination (Cole-Tobian and King, 2003) and genetic differentiation (Holsinger and Weir, 2009) where the latter reflects on the population history, including population bottlenecks (Reviewed in Greenberg *et al.*, 2008). Thus, the knowledge of the parasite's population history and its genetic diversity is important for a full understanding of the epidemiology of malaria in different populations. A previous study done by our group has shown that the signature of diversifying selection was seen most strongly in Domain II of PvAMA-1 (Gunasekera *et al.*, 2007).

Therefore, the present molecular-epidemiological study was designed to characterize the prevailing genetic diversity of domain II *Pvama-1* in Sri Lanka, using 64 *P. vivax* field isolates that included the 23 clinical isolates previously examined (Gunasekera *et al.* 2007)

from two malaria endemic areas, Anuradhapura (N=10) and Kataragama (N=28) and a non-endemic area, Colombo (N=26). Due to the low number of sequenced isolates from Anuradhapura, these isolates were combined with those from Kataragama and labelled as isolates originating from the endemic area (EA). Based on the sequences obtained, the objectives of the analysis were as follows:

1. To analyze the population genetic indices (nucleotide diversity, haplotype diversity, positive selection, recombination, linkage disequilibrium, etc.) among the three local study areas segregated as the endemic area (Anuradhapura and Kataragama) and the non-endemic area (Colombo).
2. To investigate the indices acting upon the genetic diversity on domain II of *Pvama-1* among the total *P. vivax* Sri Lankan test population, and to compare the obtained data with previously published domain II sequences from geographically diverse populations, to gain information on the global genetic diversity on domain II *Pvama-1*.
3. To compare the genetic structure of *P. vivax* and *P. falciparum*, the two parasite populations sharing the same human hosts, by drawing comparisons between the domain II of *Pvama-1* and *Pfama-1*.
4. To determine the evolutionary relationship of *Pvama-1* in local and worldwide field isolates by phylogenetic analysis.
5. To theoretically predict possible B and T cell epitopes spanning the domain II of PvAMA-1 and to evaluate the possibility of the binding ability of these epitopes with respect to occurrence of polymorphic substitutions in the domain.

Moreover, as the signature of diversifying selection was seen most strongly in domain II of PvAMA-1 from *P. vivax* isolates from Sri Lanka (Gunasekera *et al.* 2007), it was important to study the natural antibody responses against this particular domain. Moreover, as reported by Pizzaro *et al.* (2005) the invasion-inhibitory 4G2 epitope present in the domain II loop of PvAMA-1 confirmed the presence of a conformational B cell epitope that elicit a protective immune response in this region. Therefore, from a vaccine point of view, it would be

practical to include the region corresponding to the 4G2 epitope for a vaccine based on PvAMA-1.

The present cross sectional immuno-epidemiological study was designed to characterize the naturally acquired antibody responses against PvAMA-1 domain II in individuals acutely symptomatic to *P. vivax* infections in Sri Lanka. Subsequent to predicting linear B cell epitopes on PvAMA-1 domain II, seven peptides (P01 – P07) were synthesized, where peptides P04 and P05 represented the domain II loop (corresponding to the amino acid positions 294-334 of the Salvador I strain of PvAMA-1). The main objectives of the current study were as follows:

1. to determine the total (IgM + IgG) and isotype (IgM and IgG) specific antibody responses against the seven synthetic peptides of PvAMA-1 domain II by carrying out an indirect microplate Enzyme-Linked Immunosorbant Assay (ELISA), in *P. vivax* infected acute patients from a malaria non-endemic area, Colombo, and from two malaria endemic areas, Anuradhapura and Kataragama, who were previously assayed positive against the recombinant Pv66/AMA-1 protein (Wickramarachchi *et al.* 2006).
2. To evaluate the ensuing strain-specific immunity against PvAMA-1 domain II, PvMSP-1₄₂ by aligning the antibody responses with their homologues *P. vivax* amino acid sequences.

The 64 PvAMA-1 domain II amino acid sequences of clinical isolates from Sri Lanka generated 11 amino acid point mutations that gave rise to 21 amino acid haplotypes, where all 21 haplotypes were identical to the Salvador I strain from amino acid positions 294-334 which represented the domain II loop corresponding to peptides P04 and P05, while the rest of the peptides on either side of the domain II loop consisted amino acid polymorphic residues (chapter 3). Therefore, to assess possible strain-specific immunity to PvAMA-1 domain II, fifty three of the 64 PvAMA-1 domain II amino acid sequences were aligned with the homologous anti-PvAMA-1 domain II antibody responses (IgM + IgG) assayed by an indirect ELISA against each of the seven synthetic peptides, P01-P07. The seven peptides resembled the Salvador I allele types of PvAMA-1.

5.3. Results

5.3.1. Genetic diversity of *Pvama-1 domain II* within Sri Lanka

Analysis within the endemic (EA) and non-endemic areas (NEA) revealed 15 and 16 polymorphic sites giving rise to 16 and 17 point mutations, respectively. Of these, five singleton variable sites, and a parsimony informative site with three polymorphisms (nucleotide position 1025) were common to both areas. Thirteen and 14 non-synonymous changes from the EA and NEA, were observed, respectively, while 3 synonymous changes each were common to both the study areas.

Nucleotide diversity (π) was elevated in NEA ($\pi = 0.0105$ S.D. = 0.0007) compared to EA ($\pi = 0.0088$ S.D. = 0.0006) (Table 5.1) as well as the average number of nucleotide differences (k) in NEA (k= 4.323) compared to EA (k=3.60).

Differences between non-synonymous (Dn) and synonymous (Ds) mutations in both the endemic (0.0088 ± 0.0037 , $P < 0.05$) and non-endemic (0.0059 ± 0.0056 , $P > 0.05$) areas suggested stronger diversifying selection acting in the EA (Table 1). The Tajima's D statistics were found to be -0.10432 ($P > 0.01$) and -0.17592 ($P > 0.01$) for both the NEA and the EA, respectively, showing no deviation from neutrality (Table 5.1).

The McDonald-Kreitman test using *P. cynomolgi* as the out group compared the relative frequency of non-synonymous nucleotide substitutions within the species, to the relative frequency of fixed differences between species. This analysis provided evidence for departure from neutrality in the non-endemic area, where the intraspecific fixed differences were comparatively high to that of interspecific non-synonymous differences indicating a significant purifying selection acting in the NEA (Fisher's exact test, $P < 0.001$). On the contrary, both inter and intraspecific non-synonymous differences were equal in the endemic area (Table 5.2).

The minimum number of recombination events between adjacent polymorphic sites (Rm) was 3 and 2 among the EA and the NEA, respectively, whereas the recombination parameter (R) between adjacent sites and per gene was 0.1093, 44.8 for EA and 0.2002, 82.1 for NEA.

Two of the three recombination events occurred between polymorphic sites 737 / 930 and 930 / 977 in both areas. An F_{ST} value of 0.0033 (which ranged between 0-1) was observed between the endemic and the non-endemic areas suggested hardly any genetic differentiation between the two study areas.

5.3.2. Genetic diversity of *Pvama-1* domain II in Sri Lanka and geographically different countries

Analysis of the 64 *Pvama-1* sequences of Sri Lankan isolates revealed 16 polymorphic positions with a single singleton site and 15 parsimony informative sites giving rise to 17 point mutations. A nucleotide diversity (π) of 0.0095 ± 0.0005 S.D. was recorded with an average number of pairwise nucleotide difference (k) of 3.901 and a haplotype diversity (H_d) of 0.928 ± 0.015 S.D. (Table 5.1). Comparison of these results with previously published isolates from India (Thakur *et al.* 2008) indicated that the *Pvama-1DII* gene in Sri Lankan isolates were highly diverse.

The difference between D_n and D_s in the entire Sri Lankan population illustrated a significant positive selection acting on this domain ($D_n-D_s= 0.0075 \pm 0.0043$ S.D.; Z test $P < 0.05$) which confirmed previous observations by Gunasekera *et al.* (2007). Though not significant ($P > 0.10$), Tajima's test of neutrality also demonstrated a positive deviation from neutrality (Table 5.1). Data obtained by the McDonald Kreitman test which is summarized in Table 3.2.2 was suggestive of purifying selection acting on this domain due to excess intraspecific non-synonymous fixed differences (14) compared to interspecific differences between *P. vivax* and *P. cynomolgi* (13).

The minimum number of recombination events between adjacent polymorphic sites (R_m) was 4, whereas the recombination parameter (C) between adjacent sites and per gene was 0.1483 and 60.8 respectively. The R_m value in the 64 Sri Lankan isolates was similar to the 3 recombination events that occurred within 61 Indian isolates, while the R_m value in the Sri Lankan isolates was low compared to 4 and 8 recombination events that occurred from 12 and 33 isolates from two surveys in Venezuelan (Ord *et al.* 2008; Thakur *et al.* 2008). However, the recombination parameter values between adjacent sites and per gene were low compared to India (0.8537) and high to that of the two surveys from Venezuela (0.009 and

0.0103). Analysis of linkage disequilibrium (LD) index (R^2) for Sri Lankan isolates indicated equilibrium inter-site linkage with increasing nucleotide distance within the *Pvama-1* sequences. A moderate ($F_{ST} = 0.1-0.15$) genetic differentiation was evident between Sri Lanka and Venezuela (F_{ST} value = 0.1472), indicating a strong genetic drift between these two countries, whereas similar F_{ST} values were calculated between Sri Lanka, India (F_{ST} value = 0.0692) and India, Venezuela (F_{ST} value = 0.0641).

Table 5.1. DNA sequence polymorphisms in the *P. vivax ama-1* Domain II antigen among Sri Lankan isolates

	π (S.D)	Ds	Dn	Dn - Ds (S.D)	(Z-test)	Tajima's Test
Entire Population (N=64)	0.0095 (0.0005)	0.0036 (0.0022)	0.0111 (0.0035)	0.00755 (0.0043)	0.0435 (P<0.05)	0.2557 (P> 0.10)
Endemic Area (N=38)	0.0088 (0.0006)	0.0019 (0.001)	0.0106 (0.003)	0.0088 (0.0037)	0.0123 (P<0.05)	-0.17592 (P>0.10)
Non-endemic Area (N=26)	0.0105 (0.0007)	0.0059 (0.0040)	0.0118 (0.004)	0.0059 (0.0056)	0.1473 (P>0.05)	-0.10432 (P>0.10)

N - number of sequences

π - nucleotide diversity with standard deviation (SD)

Ds - rate of synonymous mutations per synonymous sites

Dn - rate of non-synonymous mutations per non-synonymous sites, using the Nei and Gojobori method

Dn - Ds - difference of Dn and Ds with their standard deviation, estimated by bootstrap with 1000 pseudo replicates

Z-test - to test significance (Nei and Kumar, 2000)

Tajima's D test - detection of departure from the neutral model.

Table 5.2. McDonald-Kreitman analyses of *Pvama-1* Domain II between *P. vivax* and *P. cynomolgy* across different countries

	Polymorphic changes within <i>P. vivax</i>		Fixed differences between species		Neutrality Index ¹	Fisher's Exact Test ²
	Synonymous	Non-synonymous	Synonymous	Non-synonymous		
EA (N=38)	3	13	31	13	10.33	0.0008***
NEA (N=26)	3	14	31	13	11.128	0.000358***
Sri Lanka (N= 64)	3	14	31	13	11.128	0.000358***
Venezuela (N=73)	2	5	31	14	5.536	0.084893
India (N=61)	4	13	31	13	7.75	0.001346**

¹ = Neutrality index indicates the extent to which the levels of amino acid polymorphism depart from the expected in the neutral model.

² = Fisher's Exact Test. * 0.01 < P < 0.05; ** 0.001 < P < 0.01; *** P < 0.001

5.3.3 Classification of *Pvama-1* haplotypes and sequence variation observed at domain II

All 64 sequences were identical between the amino acid positions 295–334, while 11 a.a. variant positions in PvAMA-1DII defined 21 a.a. haplotypes. Amino acid haplotype H1 was found to be predominant (17.2%, N=11) in Sri Lanka, and was 100% identical to the Salvador I strain (Table 5.3.2). Haplotype H9 represented 9 isolates, whereas H5 and H13 in 6, H3 and H4 in 5 isolates each were recorded. While H6 and H16 were represented by 3 isolates each, H2, H12 and H14 consisted of 2 isolates each. The remaining haplotypes represented a single isolate each (Table 5.3).

Observation of the haplotype distribution among the three study areas revealed 07 haplotypes in Anuradhapura, whereas Kataragama and Colombo manifested 13 and 14 haplotypes, respectively, (Table 5.4). Two haplotypes from Anuradhapura, 4 from Kataragama and 5 from Colombo were exclusively restricted to those particular areas. With an exception of two isolates representing H2 from Anuradhapura, all other haplotypes corroborated a single isolate. Nonetheless, haplotypes H1, H3 and H5 were present in all three test areas, H4 was exclusive to the two endemic areas. Haplotype H6 was distinctive to isolates from Anuradhapura and Colombo, while H9, H12, H13 H14 and H16 were restricted to those from Kataragama and Colombo. The predominant haplotype H1 was distributed in all three test areas representing 01, 06 and 04 isolates from Anuradhapura, Kataragama and Colombo, respectively (Table 5.4).

Alignment of the *Pvama-1* Sri Lankan sequences with those of previously published from India and Venezuela resulted in 44 nucleotide haplotypes which gave rise to 33 amino acid haplotypes (Table 5.3). Haplotypes H14 and H16 were common to all three countries. Haplotypes H1, H3, H4, H6, H9, H11 and H19 were exclusively common to both Sri Lankan and Indian isolates, while H8 and H21 represented isolates from Sri Lanka and Venezuela. A majority of the isolates (13.7%) represented H26, which included 03 and 24 isolates from India and Venezuela, respectively. Eleven isolates from Sri Lanka and 09 from India represented haplotype H1, which was 100% identical to the Sal I strain, whereas none of the Venezuelan isolates represented this haplotype (Table 5.3). Haplotype H8 which was represented by a single Sri Lankan isolate from Kataragama was similar to sixteen

Venezuelan isolates (Ord *et al.* 2008) (Table 5.3 & 5.4). An isolate from the Philippines (PH84) published by Cheng *et al.* (1994), was similar to an Indian isolate, while the Brisbane isolates (PVQ) (Cheng *et al.* 1994) was included among 8 previously published Indian isolates (Thakur *et al.* 2008). Only a single haplotype was distinctive to Venezuela, while 8 haplotypes were exclusive to India as reported by Thakur *et al.* (2008). Of the 21 amino acid haplotypes observed from the 64 Sri Lankan isolates, 10 were unique to the island (Table 5.3). Further, as 4 of these haplotypes (H5, H12, H13, and H20) were previously published by Gunasekera *et al.* (2007), 7 new haplotypes (H2, H7, H10, H15, H17 and H18) are reported for the first time from the present study. These included 6 isolates from the endemic area and two from the non-endemic area (Table 5.3).

Comparison of these 22 polymorphic nucleotide positions with other countries resulted in 12 amino acid changes (Table 5.3). Of the 12 polymorphic amino acid positions identified in this and previous studies (Ord *et al.* 2008; Thakur *et al.* 2008), 4 were trimorphic (K310E/N, Q338R/K, L342P/R and E343Q/D), while the rest of the codons displayed only two alternative amino acid residues and thus were dimorphic (Table 5.3). A single amino acid change found in PV4 and PV12 Indian isolates, where Valine (V) was replaced by Glutamate (E) at position 340 was not recorded in the Sri Lankan and Venezuelan isolates. Moreover, position 340 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 328 and 329, where non-synonymous changes occurred from Phenylalanine (F) to Leucine (L), and Isoleucine (I) to Phenylalanine (F) in two and one Sri Lankan isolates respectively, the rest of the Sri Lankan, Indian and Venezuelan isolates were similar to PH84, PVQ and Sal-I. Thus, amino acid positions 328 and 329 were exclusively distinct to Sri Lanka while position 340 was unique to India.

5.3.4 Phylogenetic analysis of *Pvama-1* in geographically different countries

Worldwide distribution of isolates in the phylogenetic tree revealed fewer clustering where most isolates had a very recent common origin and most of the changes were singletons (Figure 5.1). There were five groups that showed distinctive clustering of isolates. Of these, group 01 contained 3 Sri Lankan isolates, 2 from Colombo and 1 from Kataragama, while groups 2, 3 and 4 were unique to Venezuelan isolates. Group 5 comprised one isolate each

from Colombo and India. All Venezuelan isolates were dispersed closely, due to a single substitution or 2, 3 substitutions present in the nucleotide haplotype sequence. Several isolates from Sri Lanka and India were uniquely clustered around the Sal I strain, while a single isolate from India (PV8) and Colombo (CPV 4738) was present near the PH84 isolate from the Philippines. Interestingly, the amino acid sequence of PV8 was similar to PH84 while CPV4738 differed with a single substitution that occurred at amino acid position 338 from Glutamine/ Arginine (Q/R) (Table 5.3). The PVQ Brisbane isolate was clearly clustered between several Venezuelan isolates. (Figure 5.1).

Table 5.3. Amino acid polymorphisms in the *P. vivax* AMA-1 domain II among Sri Lanka (N=64), and their comparison with Indian (N=60) and Venezuelan (N=73) isolates

						2	2	2	3	3	3	3	3	3	3	3	
Haplotype	Sri Lanka	Philippine	Brisbane	India	Venezuela	1	3	4	1	2	2	2	3	4	4	4	4
						1	5	6	0	6	8	9	8	0	1	2	3
<i>P. vivax</i> AMA-1 Salvador I						G	E	G	K	K	F	I	Q	V	D	L	E
H1	11			9	-	.	.	.	.	.	.	.	.	.	.	.	.
H2 ^a	2			-	-	.	.	.	.	I	.	.	.	.	.	.	.
H3	5			2	-	.	K	E	.	.	.	.	.	.	.	.	.
H4	5			1	-	.	K	E	E	.	.	.	.	.	.	.	.
H5 ^a	6			-	-	.	.	.	.	.	.	.	K	.	.	P	.
H6	3			1	-	.	K	E	N	.	.	.	R	.	.	.	.
H7 ^a	1			-	-	.	K	.	.	I	.	.	.	.	V	P	.
H8	1			-	16	.	K	E	.	.	.	.	.	.	.	P	.
H9	9			1	-	E	.	.	.	I	.	.	.	.	.	R	D
H10 ^a	1			-	-	.	K	.	.	I	.	.	.	.	V	R	D
H11 ^a	1			1	-	.	.	.	.	.	.	.	.	.	.	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			5	6	.	K	.	.	I	.	.	.	.	.	P	.
H17 ^a	1			-	-	.	.	.	.	.	.	F	.	.	V	.	.
H18 ^a	1			-	-	.	.	.	.	.	.	.	.	.	V	.	.
H19	1			12	-	.	K	.	.	.	.	.	.	.	.	P	.
H20 ^a	1			-	-	.	K	.	N	.	.	.	R	.	.	.	.
H21	1			-	9	.	K	.	.	.	.	.	K	.	.	P	.
H22	-	1		1	-	.	K	.	N	.	.	.	.	.	.	P	.
H23	-		1	8	-	.	K	.	.	.	.	.	.	.	.	R	D
H24 ^b	-			5	-	.	K	.	.	.	.	.	.	E	.	P	.
H25 ^b	-			3	-	.	K	E	.	.	.	.	.	.	.	R	.
H26	-			3	24	.	K	.	.	.	.	.	.	.	.	.	.
H27	-			1	1	.	K	.	.	I	.	.	.	.	.	.	Q
H28 ^b	-			2	-	.	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 ^c	-			-	6	.	K	.	N	.	.	.	.	.	.	.	.

Haplotypes H1–H21 are based on the 64 isolates sequenced in the current study. Amino acid positions correspond to domain II of PvAMA-1 Salvador I sequence (Accession No Y16950).

H1-H21 represented Sri Lankan, H22-H33 Indian and H34 Venezuelan haplotypes.

^a - haplotypes exclusive to Sri Lanka

^b – haplotypes exclusive to India (Thakur *et al.* 2008)

^c – haplotypes exclusive to Venezuela (Rosalynn *et al.* 2008)

Table 5.4. Distribution of the PvAMA-1 domain II amino acid haplotypes among the three study areas of Sri Lanka

Haplotype	Study Area		
	Anuradhapura	Kataragama	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)

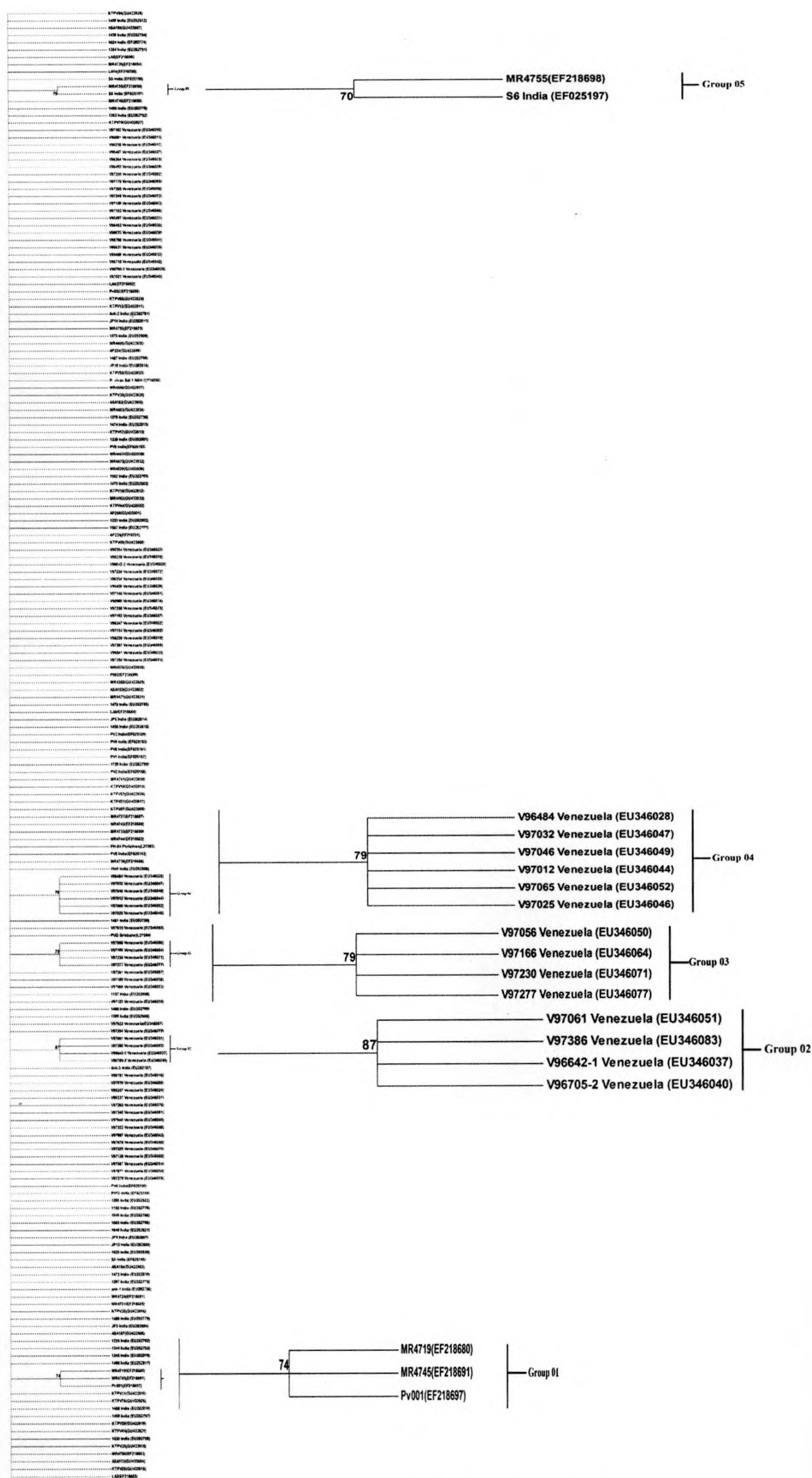


Figure 5.2. Phylogenetic tree of *Pvama-I* domain II using Kimura 2 parameter
 Isolates of Sri Lanka and other geographically different countries are included. The numbers on the node of the tree are percent bootstrap values based on 1000 pseudo- replications, only boot strap values above 70% are displayed on the tree

5.3.5 Linear T cell epitope prediction on domain II of PvAMA-1

Using the ProPred MHC class II binding peptide prediction server (Singh and Raghava, 2001), T cell epitopes were predicted to four HLA alleles; HLA-DRB1*0101, DRB1*0401, DRB1*0701 and DRB1*1101. Figure 5.2A, illustrated the T cell epitopes predicted for the domain II of PvAMA-1 Sal I strain for the four HLA alleles. Of the three polymorphic amino acid substitutions, one at amino acid position 246 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 328 from Leucine (L) / Phenylalanine (F) and 329 from Phenylalanine (F) / Isoleucine (I) were observed in all four HLA alleles. However, due to the E/G substitution, the previously predicted T cell epitope IVFEASASD to HLA-DRB*0401 was lost (Figure 5.2B). The substitution from L/F at amino acid position 328 in HLA alleles DRB*0401, 0701 and 1101 transferred the position to a obligatory P1 anchor residue, which assumed to be obligatory for high affinity binding to these HLA alleles (Figure 5.2B). Due to substitutions from L/F and F/I at positions 328 and 329 a new T cell epitope was predicted to HLA-DRB1*1101, while the predicted T cell peitope to HLA-DRB1*0101 was lost (Figure 5.2B).

A)

DRB1_0101:

RKNLGNAKFGLWVDGNCEEIPYVKEVEAKDLRECNRIVFASASDQPTQYEEEMTDYQKIQQGFRQNNREMIKSAFLPVGAFNSDNFKSKGRGFNWNFDSVKEKCYIFNTKPTCLINDINLEATTALSHPKVVPD

DRB1_0401:

RKNLGNAKFGLWVDGNCEEIPYVKEVEAKDLRECNRIVFASASDQPTQYEEEMTDYQKIQQGFRQNNREMIKSAFLPVGAFNSDNFKSKGRGFNWNFDSVKEKCYIFNTKPTCLINDINLEATTALSHPKVVPD

DRB1_0701:

RKNLGNAKFGLWVDGNCEEIPYVKEVEAKDLRECNRIVFASASDQPTQYEEEMTDYQKIQQGFRQNNREMIKSAFLPVGAFNSDNFKSKGRGFNWNFDSVKEKCYIFNTKPTCLINDINLEATTALSHPKVVPD

DRB1_1101:

RKNLGNAKFGLWVDGNCEEIPYVKEVEAKDLRECNRIVFASASDQPTQYEEEMTDYQKIQQGFRQNNREMIKSAFLPVGAFNSDNFKSKGRGFNWNFDSVKEKCYIFNTKPTCLINDINLEATTALSHPKVVPD

B)

DRB1_0101:

RKNLGNAKFGLWVDGNCEEIPYVKEVEAEDLRECNRIVFASASDQPTQYEEEMTDYQKIQQGFRQNNREMIKSAFLPVGAFNSDNFKSKGRGFNWNFDSVKKKCYIFNTKPTCLINDKNLEATTALSHPQVDLE

DRB1_0401:

RKNLGNAKFGLWVDGNCEEIPYVKEVEAEDLRECNRIVFASASDQPTQYEEEMTDYQKIQQGFRQNNREMIKSAFLPVGAFNSDNFKSKGRGFNWNFDSVKKKCYIFNTKPTCLINDKNLEATTALSHPQVDLE

DRB1_0701:

RKNLGNAKFGLWVDGNCEEIPYVKEVEAEDLRECNRIVFASASDQPTQYEEEMTDYQKIQQGFRQNNREMIKSAFLPVGAFNSDNFKSKGRGFNWNFDSVKKKCYIFNTKPTCLINDKNLEATTALSHPQVDLE

DRB1_1101:

RKNLGNAKFGLWVDGNCEEIPYVKEVEAEDLRECNRIVFASASDQPTQYEEEMTDYQKIQQGFRQNNREMIKSAFLPVGAFNSDNFKSKGRGFNWNFDSVKKKCYIFNTKPTCLINDKNLEATTALSHPQVDLE

Figure 5.2. Amino acid sequence of domain II of PvAMA-1 with the predicted T cell epitopes obtained for the HLA-DRB1*0101, HLA-DRB1*0401 and HLA-DRB1*0701 molecules.

Epitopes with polymorphic sites are highlighted in green

A) represents the epitopes on the PvAMA-1 Salvador I strain

B) represents the epitopes due to polymorphism

5.3.6. Total (IgM + IgG) antibody responses against synthetic peptides representing PvAMA-1 domain II

Peptide specific total (IgM + IgG) antibody responses were assayed using an indirect micro plate ELISA in 162 serum samples from vivax malaria patients from the three study areas, Colombo (N=56), a malaria non endemic area, and Anuradhapura (N=39) and Kataragama (N=67), two endemic areas and of a set of 28 individuals who lacked exposure to malaria (normal healthy controls).

5.3.6.1. Prevalence of anti-PvAMA-1 domain II total antibodies against seven PvAMA-1 peptides among the three study areas

The prevalence of anti-PvAMA-1 domain II total antibody responses to the seven peptides separately varied across each test area with a high responding proportion or prevalence observed for peptides P04 and P05. Peptide P04 recorded the highest antibody prevalence of 64%, 42%, and 95% from Colombo, Anuradhapura and Kataragama, respectively, while the corresponding prevalence for P05 were 25%, 74% and 70%. The antibody prevalence for peptides P01, P02, P03, P06 and P07 were 29%, 21%, 13%, 13% and 20% from Colombo, 46%, 13%, 33%, 8%, 44% from Anuradhapura and 40%, 31%, 13%, 15% and 39% from Kataragama, respectively (Table 5.5).

Further, the serum samples from Colombo were subdivided in to two groups according to their prior exposure to the infection. The previously not exposed group (PNE) consisted of those patients suffering their first malaria infection, while, the other group consisted of those who had prior exposure to the infection (PE). It was evident that PE and PNE groups responded mainly against peptide P04 with a prevalence of 61% and 73%, respectively. The lowest prevalence of 6% observed for PE was against P03, while for PNE, the lowest prevalence of 6.7% was recorded against P06 (Table 5.5). 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 to their other two counterparts (Table 5.5, Chi square test, $P < 0.05$). Although, no significant difference in prevalence were recorded between the three study areas against peptides P01, P02 and P06, a high antibody prevalence was recorded against peptides P02 and P06 in individuals from Kataragama compared to those from Anuradhapura and Colombo, while P01 in individuals from Anuradhapura (Table 5.5, Chi square test, $P > 0.05$).

Table 5.5. Prevalence of responders with acute *P. vivax* infections against PvAMA-1 domain II peptides (P01 – P07) among the three study areas

Study Area	Number of Samples	P01	P02	P03	P04	P05	P06	P07
		Prevalence (%)						
Colombo (Total)	56	29	21	13	64	25	13	20
PE ^a	36	19	14	6	61	19	11	17
PNE ^b	15	40	33	13	73	27	7	27
Anuradapura	39	46	13	33	42	74	8	44
Kataragama	67	40	31	13	95	70	15	39

^a – previously exposed to malaria

^b – previously not exposed to malaria

5.3.6.2. Prevalence of anti-PvAMA-1 domain II total antibodies to peptide combinations among the three study areas

The proportion of patients that responded to the seven synthetic peptides of PvAMA-1 domain II were further analysed by grouping them into those who i) responded to all seven synthetic peptides ii) did not respond to all seven synthetic peptides and iii) preferentially responded to one of the seven synthetic peptides (Table 5.6).

Amongst the 162 serum samples tested, only a single sample from Kataragama responded to all seven peptides, while, 9 from Colombo and one sample each from Anuradhapura and Kataragama lacked anti-PvAMA-1 antibodies to all seven peptides. Though not significant, the non-responding prevalence to all 7 peptides was high in the PE group compared to the PNE group (Chi square test, $P > 0.05$). Peptide P04 was preferentially recognised by individuals in all three test areas including the two exposure groups of Colombo. Individuals from all three test areas lacked preferential responders against P03 and P06, while preferential responders against P01 and P02 were recorded exclusively from the PE group of Colombo with responding prevalence of 8% and 2%, respectively (Table 5.6). Though, a preferential responding prevalence of 3% and 2% was exclusively observed against P07 from Anuradhapura and Kataragama, respectively, a 10% preferential responding prevalence was recorded exclusively from Anuradhapura against P05 (Table 5.6).

Total antibody responses in the three study areas against the seven peptides of PvAMA-1 domain II demonstrated that individuals tend to respond more to peptides P01, P04 and P05 (Table 5.6). Therefore, these antibody responses were further analysed by grouping them into those who i) responded to all three peptides ii) did not respond to all three peptides iii) responded preferentially to P01 and P04 iv) responded preferentially to P01 and P05 and v) responded preferentially to P04 and P05 (Table 5.6).

Accordingly, the responding prevalence against P01, P04 and P05 were significantly high from Kataragama (33%) compared to Anuradhapura (21%) and Colombo (15%), while a significantly high non-responding prevalence of 11% was observed from Colombo compared to its endemic counterparts (Anuradhapura – 5% and Kataragama – 3%) against all three peptides (Chi square test, $P < 0.05$), with the PE group recording the highest non-responding prevalence compared to the PNE group. All three study areas including the two exposure groups preferentially recognised peptide combination P01/P04, P01/P05 and P04/P05 with

high responding prevalence recording for P04/P05 exclusively from Colombo and Kataragama and for P01/P05 exclusively from Anuradhapura (Table 5.6). A prevalence of 68% recorded from Kataragama was significantly different compared to Colombo (24%) and Anuradhapura (31%) against peptide combinations P04/P05 (Chi square test, $P < 0.05$). Though not significant, a positive trend was observed in recognition of both P04 and P05 in the endemic areas (Table 5.6).

Table 5.6. Anti-PvAMA-1 total (IgM + IgG) antibody prevalence to each peptide and peptide combinations among the three study areas

Peptide combinations	Colombo (Total) (N=56)	PE ^a (N=36)	PNE ^b (N=15)	Anuradhapura (N=39)	Kataragama (N=67)
Prevalence (%)					
Responded to all 7 peptides	-	-	-	-	2
Not-responded to all 7 peptides	16	19	13	3	2
Preferentially responded to:					
P01	6	8	-	-	-
P02	2	3	-	-	-
P03	-	-	-	-	-
P04	25	28	27	3	12
P05	-	-	-	10	-
P06	-	-	-	-	-
P07	-	-	-	3	2
Responded to P01,P04,P05	15	20	8	21	33
Not-responded to P01, P04, P05	26	67	8	5	3
Preferentially responded to:					
P01/P04	18	20	14	26	38
P01/P05	16	20	11	39	33
P04/P05	24	47	8	31	68

^a – Previously exposed to malaria

^b – Previously not exposed to malaria

5.3.6.3. Comparison of anti-PvAMA-1 domain II total antibody magnitudes to the seven peptides among the three study areas

Of the seven PvAMA-1 domain II peptides, P04, P06 and P07 recorded significantly high anti-PvAMA-1 antibody magnitudes from Kataragama compared to Colombo and Anuradhapura, where the highest antibody magnitudes were recorded for P07 in all three test areas (Table 5.7). A significantly high antibody magnitude was recorded from Anuradhapura compared to Kataragama and Colombo against P05 (Kruskal-Wallis test, $P < 0.05$, Table 5.7). Magnitudes of the two exposure groups of Colombo did not significantly differ against the seven peptides (Mann-Whitney U test, $P > 0.05$), rather the PE and the PNE groups recorded high magnitudes against P01, P03, and P02, P04, P05 and P07. Further, a significantly predominant antibody magnitude was recorded from Kataragama compared to non-endemic Colombo against P01 (Mann-Whitney U test, $P < 0.05$, Table 5.7). Though not significant, an elevated antibody magnitude was recorded in Kataragama against P02, and in Colombo against P03.

A significant positive correlation was observed between the antibody magnitudes against peptides P04/P05 (Spearman correlation coefficient, $r = 0.709$) and P04/P07 ($r = 0.886$) among patients from non-endemic Colombo, ($P < 0.05$) and endemic Kataragama (P04/P05, $r = 0.36$, P04/P07, $r = 0.474$, $P < 0.01$), while, a negative correlation was observed among responders of Anuradhapura against peptides P04/P05 ($r = -0.734$, $P < 0.05$). A significant positive correlation between antibody magnitudes against P02/P07 was exclusively observed from Kataragama ($r = 0.509$).

Table 5.7. Total (IgM + IgG) antibody magnitude against peptides P01-P07 in acute *P. vivax* patients from the three study areas

Area	Antibody Magnitude													
	Peptide P01		Peptide P02		Peptide P03		Peptide P04		Peptide P05		Peptide P06		Peptide P07	
	Mean ^a	SEM ^b	Mean ^a	SEM ^b	Mean ^a	SEM ^b	Mean ^a	SEM ^b	Mean ^a	SEM ^b	Mean ^a	SEM ^b	Mean ^a	SEM ^b
Colombo (Total)	0.189	0.008	0.289	0.013	0.222	0.039	0.154	0.014	0.147	0.014	0.243	0.006	0.391	0.017
PE ^c	0.19	0.011	0.292	0.018	0.328	0.124	0.14	0.02	0.12	0.013	0.248	0.006	0.376	0.015
PNE ^d	0.179	0.014	0.294	0.025	0.177	0.014	0.154	0.018	0.161	0.031	-		0.425	0.037
Anuradhapura	0.232	0.017	0.279	0.026	0.2	0.007	0.18	0.052	0.394	0.041	0.312	0.036	0.439	0.015
Kataragama	0.239	0.015	0.299	0.016	0.209	0.009	0.22	0.021	0.286	0.025	0.338	0.024	0.567	0.04

^a - The mean absorbance_{490nm} 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 level of the anti-PvAMA-1 total antibody response to each peptide in each individual.

^b - Standard Error of Mean

^c - Previously exposed to malaria

^d - Previously not exposed to malaria

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

Attempts were made to establish associations between total (IgM + IgG) antibody magnitudes and responding prevalence with different host factors (*i.e.* age of patient, parasitaemia and the number of past infections) using bivariate Spearman correlation tests and Chi square test for linear trend.

All three test areas lacked association either 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 magnitudes 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 evident between previous exposure with antibody magnitudes against P05 in patients from Anuradhapura (Spearman correlation coefficient = 0.532, $P < 0.01$). Majority of the responders against P05 were those who either lacked previous exposure to the infection, or who had a exposure of 1-2 infections only.

For further analysis of 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. 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, $P > 0.05$) (Figure 5.3).

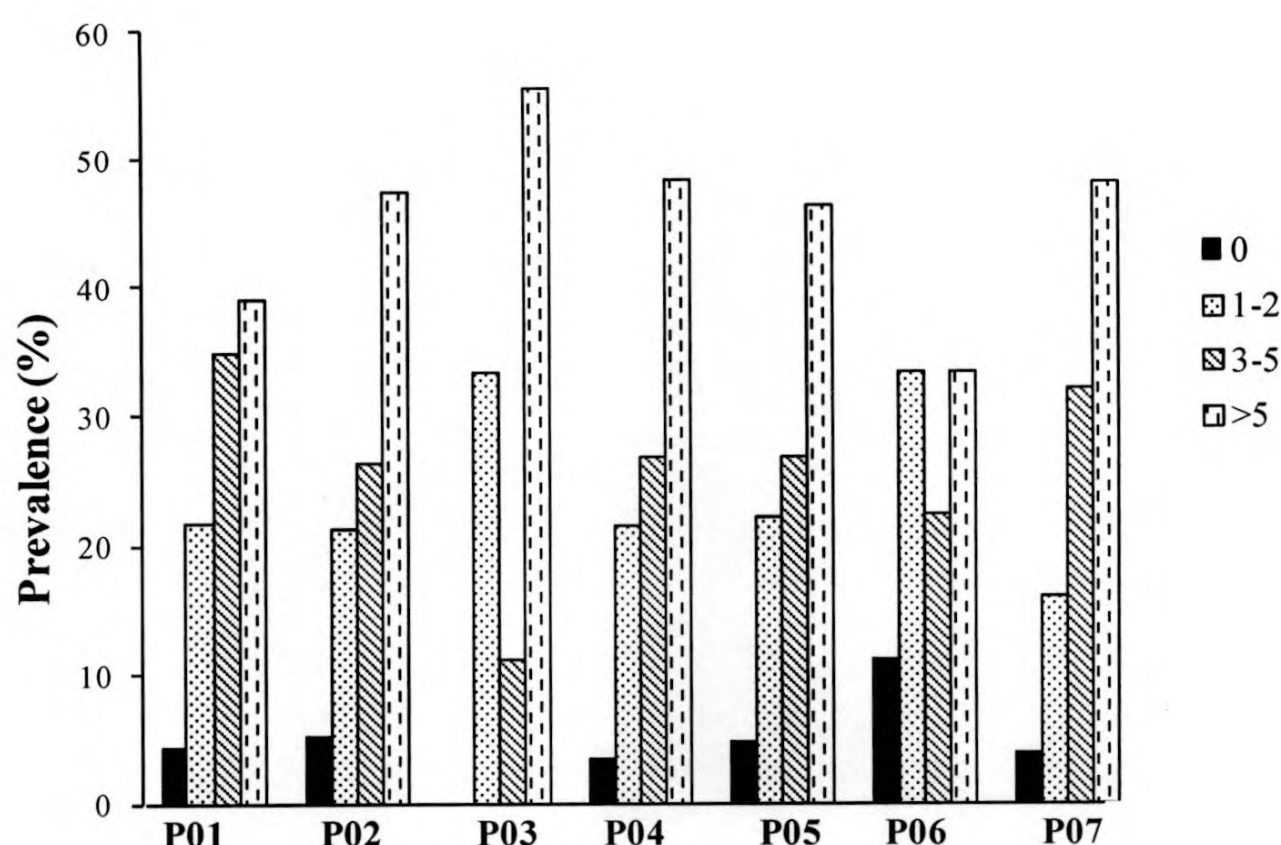


Figure 5.3. Antibody prevalence against the synthetic peptides of PvAMA-1 domain II with different malaria exposure groups in Kataragama

Each past exposure group is indicated is indicated in the legend.

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

As the total antibody responses to each of the seven peptides of PvAMA-1 domain II in the study areas revealed that individuals tend to respond more to peptides P04 and P05, these two peptides were further subjected to the analysis of isotype specific IgM and IgG subclasses antibody responses. Serum samples positive for the total (IgM + IgG) antibody response against P04 and P05 from Colombo (N=36), Anuradhapura (N=33) and Kataragama (N=65) were assayed by sandwich antibody ELISA to study IgM and IgG subclass specific antibody responses against both P04 and P05.

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

The prevalence of anti-PvAMA-1 IgM antibody response in Colombo was 6% and 8% against P04 and P05, respectively. Prevalence of 18% and 86% from Anuradhapura, 11% and 14% from Kataragama, were recorded against P04 and P05, respectively, with the IgM responding prevalence from Anuradhapura being significantly higher than that of both Colombo and

Kataragama, exclusively to P05 (Chi square test, $P < 0.001$, Table 5.8). Lack of anti-PvAMA-1 IgM antibody response was observed in the PNE group for P04 and in the PE group for P05.

The prevalence of anti-PvAMA-1 IgG antibody response in Colombo was 9% and 15% against P04 and P05, respectively. Prevalence of 18% and 38% from Anuradhapura, 34% and 13% from Kataragama, were recorded against P04 and P05, respectively, with significantly elevated responding prevalence recording from Kataragama against P04 and Anuradhapura against P05 (Chi square test, $P < 0.05$). Lack of anti-PvAMA-1 IgG antibody responses were observed in both the exposure groups from Colombo to P05, while the PNE group exclusively lacked an IgG antibody response against P04 (Table 5.8).

5.3.7.2. Comparison of anti-PvAMA-1 isotype antibody prevalence against peptides P04 and P05

Individuals from Anuradhapura recorded significantly elevated IgM antibody prevalence against P05 compared to P04, conversely, individuals from Kataragama recorded significantly elevated IgG antibody prevalence against P04 compared to P05 (Chi square test, $P < 0.05$, Table 5.8). Though, patients from Kataragama, recorded significantly predominant IgG antibody prevalence compared to IgM against P04, individuals from Anuradhapura recorded the reverse against P05 where approximately twice the prevalence of IgM antibodies (86%) were detected compared to that of IgG antibodies (38%) (Chi square test, $P < 0.01$, Table 5.8).

Table 5.8. Prevalence of isotype specific IgM and IgG responders of acute *P. vivax* infection against PvAMA-1 domain II peptides P04 and P05 among the three study areas

Area	Number of Samples (N)	Prevalence (%)			
		Peptide P04		Peptide P05	
		IgM	IgG	IgM	IgG
Colombo (Total)	36	6	9	8	15
PE ^a	21	10	10	0	0
PNE ^b	12	0	0	25	0
Anuradhapura	33	18	18	86	38
Kataragama	65	11	34	14	13

^a – Previously exposed to malaria

^b - Previously not exposed to malaria

5.3.7.3. Comparison of anti-PvAMA-1 domain II antibody isotype magnitudes against peptides P04 and P05 among the three study areas

The IgM isotype antibody magnitude against P05 was significantly lower compared to P04 in all three study areas (Mann Whitney U test, $P < 0.05$, Table 5.9). While an elevated IgG antibody magnitude was recorded from Anuradhapura against P05 compared to P04, the other two study areas recorded the reverse (Mann Whitney U test, $P > 0.05$, Table 5.9).

A significantly elevated IgM antibody magnitude was recorded from Anuradhapura compared to Colombo and Kataragama against P05 (Kruskal-Wallis test, $P < 0.05$), while, though not significant a similar result was observed for IgM antibody magnitudes against P04. Further, a significantly elevated IgG antibody magnitude was recorded from Kataragama against P04 (Kruskal-Wallis test, $P < 0.05$) and though not significant from Anuradhapura against P05 compared to the other two study areas ($P > 0.05$, Table 5.9).

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

Individuals from Anuradhapura and Kataragama showed a negative trend between anti-IgG responding prevalence against P05 (Chi square for linear trend, 4.21, $P < 0.05$) and anti-IgM responding prevalence against P04 ($P > 0.05$), respectively, with increasing exposure. Individuals from Colombo lacked any isotype prevalence trend against P04 or P05 with increasing exposure to the infection. In order to explore the variation of anti-PvAMA-1 IgM and IgG isotype prevalence with previous exposure to malaria, patients in the two endemic test areas were grouped together and were compared with those of the non-endemic area. The past exposure group of the pooled endemic and non-endemic categories were further divided into two groups; individuals with i) IgM restricted response and a combination of IgM and IgG response, and ii) IgG restricted response. In endemic regions, the group with no previous malaria exposure to P05 exhibited only an IgM restricted and a combination of IgM with IgG response with a 100% antibody prevalence, while individual samples were absent for PNE group of P04 (Figure 5.4 A and C). Further, in the PE groups of P04, with increasing exposure the prevalence of IgM restricted and a combination of IgM + IgG responses were reduced and were minimal in those with more than 5 past malaria infections. In parallel, prevalence of the IgG restricted response increased with exposure and was maximum in patients with more than 5 past malaria infections (Chi squared for linear trend 0.096, $P = 0.757$, Figure 5.4A). Similarly, the prevalence of the IgM and a combination of IgG and IgM restricted responses against P05 decreased with exposure (except those with 1-2 past infections) and in patients with no previous infections only IgM restricted and a combination of IgM and IgG restricted response was observed. In parallel, the IgG restricted response increased with exposure (Chi squared for linear trend 0.046, $P = 0.83$, Figure 5.4C). Therefore, a shift of anti-PvAMA-1 domain II isotype specific antibody response against P04 from IgM and combination of IgG and IgM to functionally important IgG isotype specific antibody responses was evident with concurrent increase of past exposure to malaria in endemic areas. A similar pattern of subclass-specific shift towards cytophilic IgG antibodies dominated the P05 antibody response with increasing exposure in endemic residents ($P > 0.05$), although this phenomenon was less marked than the P04 specific switch to cytophilic IgG.

Table 5.9. Anti-PvAMA-1 domain II antibody isotype magnitudes in acute *P. vivax* patients against peptides P04 and P05 from the three study areas

Area	Peptide P04				Peptide P05			
	Magnitude of IgM		Magnitude of IgG		Magnitude of IgM		Magnitude of IgG	
	Mean ^a	SEM ^b	Mean	SEM	Mean	SEM	Mean	SEM
Colombo (Total)	0.134	0.0007	0.249	0.006	0.084		0.163	0.002
PE ^c	0.134	0.0007	0.246	0.009	-		-	
PNE ^d	-		-		0.084		-	
Anuradhapura	0.178	0.024	0.262	0.009	0.13	0.008	0.292	0.042
Kataragama	0.155	0.008	0.311	0.012	0.096	0.005	0.24	0.022

^a - The mean absorbance_{490nm} obtained at a serum dilution of 1:15 for P04 and P05 was considered a measure of the level of the anti-PvAMA-1 IgM and IgG antibody response of each individual. The cut-off value was the mean OD_{490nm} value plus 2 standard deviation of the normal healthy individuals from Colombo with no past history of malaria.

^b - Standard Error of the Mean

^c - Previously exposed to malaria

^d - Previously not exposed to malaria

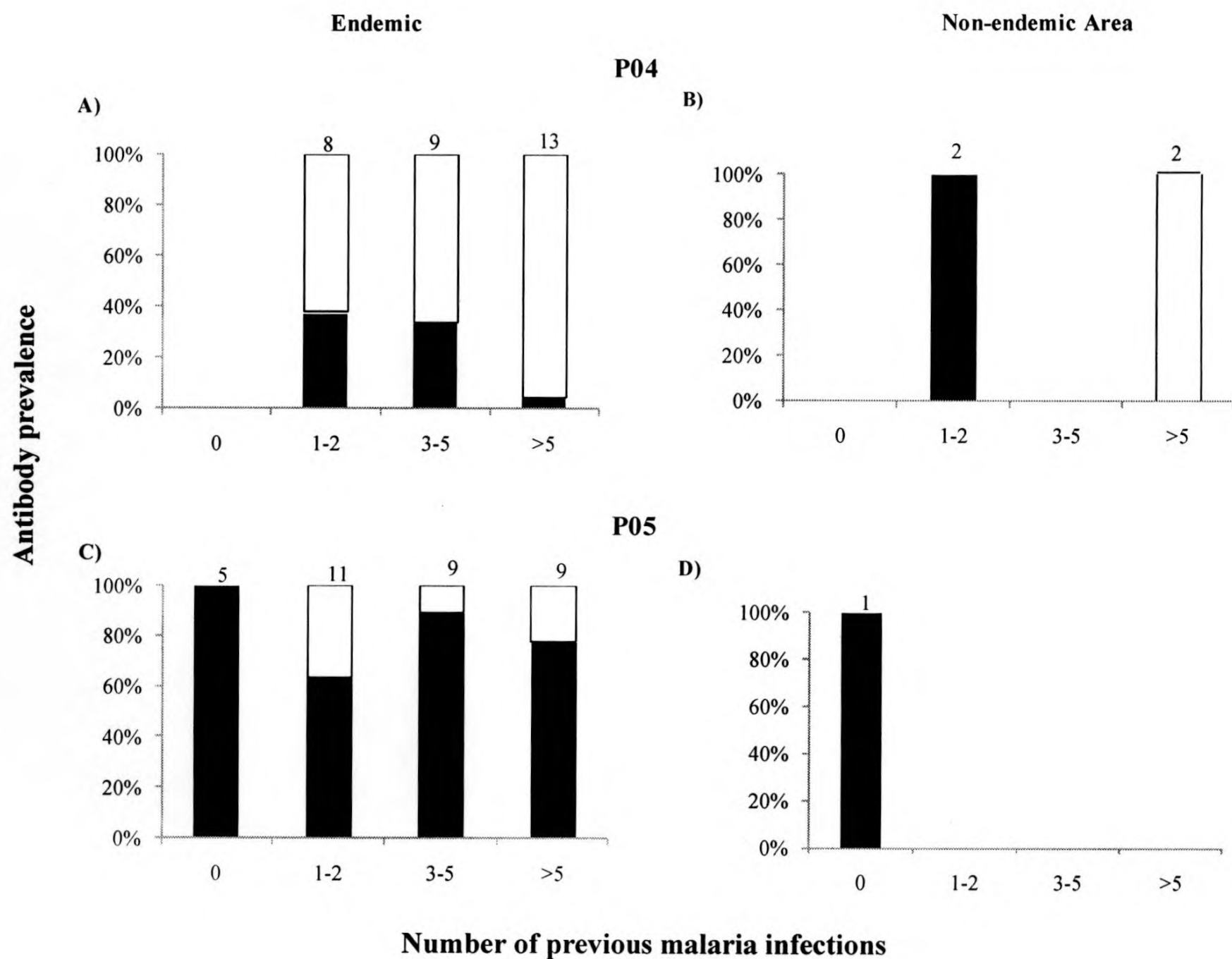


Figure 5.4. Pattern of anti-PvAMA-1 isotype-specific antibody prevalence with previous exposure to malaria

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.

5.3.8. Strain-specific immunity to PvAMA-1 domain II

Of the 64 amino acid sequences of PvAMA-1 domain II clinical isolates, 53 produced diverse amino acid haplotypes to the seven synthetic peptides separately, due to single amino acid substitutions on domain II of PvAMA-1, where a majority of haplotypes (N=12) were produced for P07. As, P04 and P05 were identical to the PvAMA-1 Salvador I strain, these two peptides generated a single amino acid haplotype each. The H1 amino acid haplotype generated in all seven peptides was identical to the PvAMA-1 domain II Salvador I strain.

While, the anti-PvAMA-1 domain II antibody prevalence of individuals representing amino acid haplotypes of P01 and P03 decreased with reducing homology to the Sal I strain (Chi square test, $P>0.05$), the antibody prevalence against P02 increased though, the homology of individuals of haplotypes to Sal I (P2H1 - 100%, P2H2 - 92% and P2H3 - 96%) varied. Nevertheless, amino acid haplotype homology percentages were similar (98%) in P6H2 and P6H3 of P06, P6H2 lacked anti-PvAMA-1 antibodies and P6H3 recorded elevated anti-PvAMA-1 antibody prevalence compared to P6H1 against P06 (Chi square test, $P>0.05$) (Figure 5.5).

Though, the 52 amino acid sequences of clinical isolates were identical to the PvAMA-1 domain II peptide construct of P04 and P05, the anti-PvAMA-1 domain II antibody prevalence was restricted to 82% and 80% against P04 and P05, respectively. Importantly, majority of responders against P04 and P05 included individuals from endemic Kataragama (46% for P04, 40% for P05), while 67% and 63% were exposed to the infection 3 times or more for P04 and P05, respectively. A single individual with no previous malaria infection recorded anti-PvAMA-1 domain II antibodies against both P04 and P05 and represented the amino acid sequence identical to the Sal I strain. Moreover, of the 18 % and 20% non-responders against P04 and P05, a majority (11%) were residents of malaria non-endemic Colombo. Of the 5 individuals who lacked anti-PvAMA-1 antibodies against both P04 and P05 from Colombo, 2 lacked antibodies against all 7 peptides. Furthermore, 2 individuals harbouring *P. vivax* parasites with amino acid sequence identical to the Sal I PvAMA-1 domain II strain, and with previous exposures of 0 and 1, recorded a 100% and a 0% anti-PvAMA-1 domain II antibody prevalence against both P04 and P05, respectively.

The amino acid sequence homology to P07 ranged from 100-84%, where individuals representing amino acid haplotype P7H1 with a 100% homology to the PvAMA-1 domain II Sal I strain was restricted to a 31.6% anti-PvAMA-1 domain II antibody prevalence (Figure 5.5). Further, anti-PvAMA-1 domain II antibody prevalence against P07 varied to haplotypes with identical homology percentage (but with dissimilar amino acid sequences) to the Sal I PvAMA-1 domain II; for eg. antibody prevalence of 0 to P7H2, P7H4, P7H9 and P7H12 and 100 to P7H6 (% homolog of 96%), 0-100% to P7H3, P7H10 and P7H11 (92%), and 0-37% to P7H5 and P7H7 (88%) was recorded. This may imply strain-transcending immune responses directed against P07 of PvAMA-1 domain II.

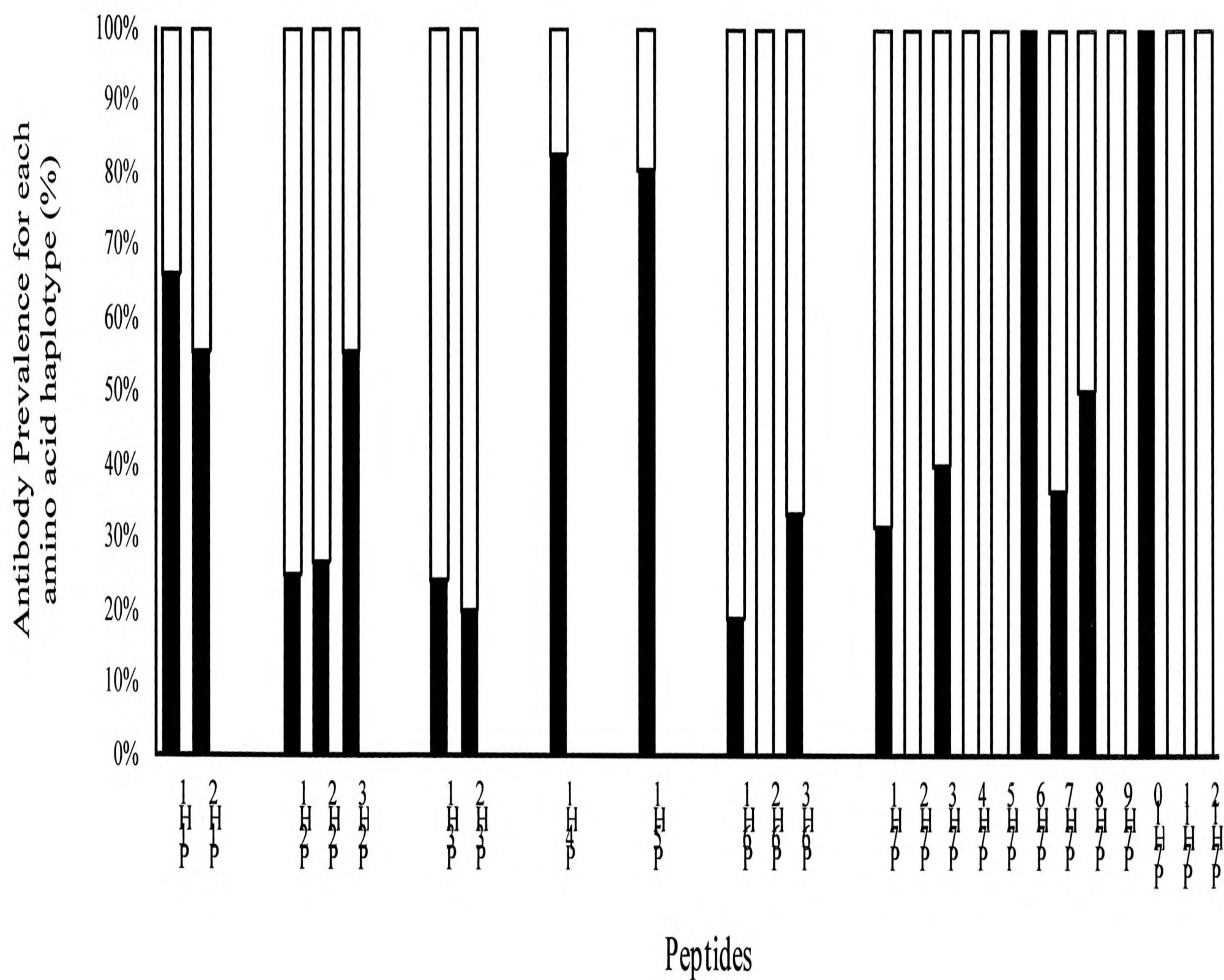


Figure 5.5. Distribution of the amino acid haplotypes to each of the seven synthetic peptides P01-P07 representing PvAMA-1 domain II, and the anti-PvAMA-1 domain II antibody prevalence against the seven synthetic peptides

Each bar represents, responders (■) and non-responders against each synthetic peptide (□)

5.4. Discussion

The diversity of the *Pvama-1* domain II in the *P. vivax* population circulating in endemic areas of Sri Lanka, where malaria is considered to be unstable with low transmission was extensively investigated in the current study.

High nucleotide diversity (π) among the isolates from the non endemic area (NEA) is plausible as the origin of these isolates were traced back to 11 different administrative districts of the island (Ampara, Anuradhapura, Hambantota, Jaffna, Kurunagala, Monaragala, Mullaitivu, Pollonnaruwa, Puttalam, Ratnapura and Vavuniya), which represented both the intermediate and the dry zones including the endemic test areas in the current study.

Though positive selection was acting on both the endemic areas (EA) and the non endemic area (NEA), the difference between Dn-Ds was not significant in the NEA suggesting that the EA had a tendency of stronger diversifying selection acting on PvAMA-1DII presumably to avoid host immune pressure, while the latter deviated from neutrality to a lesser extent. Therefore, the NEA demonstrated purifying selection on this domain which is further conferred by the McDonald test which revealed significantly high intraspecific fixed differences of *P. vivax* in the NEA area.

High nucleotide diversity of *Pvama-1DII* in the Sri Lankan clinical isolates compared to those from India (Thakur *et al.* 2008) was further reiterated by the sliding window option with a nucleotide diversity ranged from 0.007-0.026 and 0.004-0.02, respectively. Moreover, similar haplotype diversity results were obtained from Sri Lanka and India compared to Venezuela (Thakur *et al.* 2008, Ord *et al.* 2008), which coincided with the number of amino acid haplotypes obtained from Sri Lanka (21), India (20) and Venezuela (8). The comparison of *Pvama-1* sequence divergence of Brazilian isolates with those from Asia, Africa, and Oceania, demonstrated that sequence divergence in Brazil was lower compared with different old world populations (Greenberg *et al.* 2008). This confirms that nucleotide diversity (π), polymorphic sites and the average number of pairwise nucleotide difference (k) in the old world *P. vivax* population (i.e. Sri Lanka and India) was high in comparison to the new world population (i.e. Venezuela).

Genetic polymorphism in *ama-1* is maintained by two evolutionary forces (Escalante *et al.* 2001); i) recombination and ii) natural selection. Evaluating intragenic recombination is

important since new combination of epitopes could be thus generated. The recombination parameter (C) and frequency of recombination events (Rm) in Sri Lankan *P. vivax* isolates indicated that meiotic recombination occurring between the adjacent polymorphic sites were responsible for high allelic diversity at domain II of *Pvama-1* (Gunasekera *et al.* 2007). However, linkage disequilibrium of the present study showed a significant linkage across the domain II of the *Pvama-1* sequences. The very low value of the *Pvama-1* recombination parameter and the observed maintenance of significant linkage suggest very little meiotic recombination occurs in this gene in Sri Lanka. This was further reiterated by the 21 amino acid haplotypes generated, due to 11 amino acid substitutions. Interestingly, amino acid haplotype H9 was common to Sri Lanka and India, where the origin of the Sri Lankan isolate was traced back to a resident in Colombo who had visited India and had contracted the disease. These observations 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 (Gunasekera *et al.* 2007). The latter was evident as amino acid haplotypes H8, H14, and H16 consisted isolates common to Venezuela and Sri Lanka.

Though, the genetic differentiation among *Pvama-1* domain II of Sri Lankan patients was very low due to diminutive F_{ST} values between the three study areas of Sri Lanka, there were only 3 amino acid haplotypes that were common to all three test areas. Amino acid haplotype H1 which was highly prevalent in Sri Lanka was identical to the domain II of PvAMA-1 Sal I strain prevalent in India as well. Further, F_{ST} value between India and Sri Lanka suggested low genetic differentiation between the two populations and was supported by the majority of common haplotypes among the two countries. As to be expected, the genetic differentiation between Sri Lankan and Venezuelan isolates was relatively high, which was confirmed by the unique clustering of some Venezuelan isolates. Thus, *Pvama-1DII* shows strikingly less geographical differentiation within Sri Lanka than between Sri Lanka and Venezuela. An alternative hypothesis to account for the reduced polymorphism in Sri Lankan *Pvama-1* sequences may be that selection at this locus has been relaxed as mentioned by Greenburg and co-workers, (2008). However, the most frequently observed haplotype in India and Venezuela differed from the Sal I strain due to 2 and a single amino acid polymorphism, respectively. Further fixation index values indicated similar genetic differentiation between Sri Lanka / India and India / Venezuela, which could be justified as there were four haplotypes common among Venezuela and India where an intermediate gene flow could occur compared to the low gene flow between Sri Lanka and Venezuela.

The phylogenetic tree constructed using geographically different areas worldwide including those isolates from this study, revealed fewer clustering where most isolates had a very recent common origin and most of the changes were singletons. A study by Greenburg *et al.* (2008) suggesting the hypothesis that the alleles established in the new world represented a sample of worldwide genetic diversity at the *Pvama-1* locus was clearly observed by the current study where some Venezuelan isolates though clustered uniquely did not cluster separately from old world sequences of Sri Lanka and India. There was evidence of reduced genetic diversity at the *Pvama-1DII* 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 (Greenburg *et al.* 2008). This is due to the reflecting effects of recent spread of the parasite, whereas those from the old world appear to reflect a very ancient selectively maintained polymorphism.

Positive selection acting on PvAMA-1DII in Sri Lankan clinical isolates suggests that this region is under biological functional constraints as well as being targets of host immune mechanisms. Sequence analysis of the protein demarcated a number of polymorphic residues indicative of immune evasion. Some of these polymorphic sites were enclosed in T cell epitopes in the present analysis, which in turn predicted an epitope that did not exist while a new epitope was predicted separately, to two MHC alleles respectively, demonstrating that malaria parasite generates immune evasive ‘smoke-screen epitopes’ to mislead the identification by the host immune system (Reviewed in Mannan *et al.* 2003). However, the B cell epitope predicted in the PvAMA-1 domain II loop showed a very low binding score highlighting that the probability of the epitope being linear was highly unlikely. This was confirmed 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 (Pizzaro *et al.* 2005). This B cell epitope overlaps with a highly immunogenic T helper cell epitope on the sequence (Lala *et al.* 1996). The location of the 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. The loop is strictly conserved in *P. vivax* with no polymorphic sites detected in all local and global isolates in this study, suggesting that functional constraints may operate to prevent antigenic variation in this surface exposed segment.

In conclusion, the present study demonstrated the diversifying nature of the *P. vivax ama-1* domain II probably due to host immune pressure. The phylogenetic study revealed the

relatedness of Sri Lankan clinical isolates with that of neighbouring India, and evolutionary path of the old world population to the new world. It was also noted, that though the nucleotide diversity was high among isolates collected from residents of Colombo, they were not under a significant positive selection indicating that due to the lack of long term malaria transmission in Colombo, the sustainability of different alleles/strains will not be present in the area for a long period of time. Polymorphism occurring in T and B cell epitopes and generation of unique haplotypes to each country can be a factor limiting proper vaccine effectiveness. However, in depth analysis of the immunological importance of the domain II loop of PvAMA-1 is imperative as this region is conserved among *P. vivax* and *P. falciparum* species as well as it practically demonstrated the presence of a conformational epitope indicative of a functional importance to the protein. Finally the region corresponding to the domain II loop will be an ideal vaccine candidate to be used in a combined subunit vaccine in the future.

Humoral immune responses against blood stage antigens are believed to be an important component of naturally acquired immunity to *Plasmodium* (Marsh and Kinyanjui, 2006; Yazdani *et al.* 2006). Immune response to a challenge infection is readily induced in humans and other animals by clinically induced or laboratory induced blood infections, respectively, followed by drug cure when the challenge infection is with parasites of the same stock as the strain used for immunization. However, when challenge infections are with parasites of a different genetic origin, albeit of the same species, the degree of protection against the blood parasites and the disease symptoms that they cause is generally significantly less (Reviewed in Cheesman *et al.* 2006). These observations have led to the concept of “strain-specific” protective immunity in malaria, according to which each naturally experienced inoculum of malaria parasites usually contains a characteristic and genetically determined set of target antigens of protective immunity.

Peptides P04 and P05 appeared to be highly immunogenic in individuals from both malaria endemic and non-endemic areas of Sri Lanka. The homologous amino acid sequences of P04 and P05 was identical to the recombinant PvAMA-1 Sal I strain and existed in the domain II loop, which is conserved among *Plasmodium* species (Chesne-Seck *et al.* 2005). Characterisation of antibody responses against AMA-1 in malaria exposed individuals from Papua New Guinea using different allelic forms of the entire AMA-1 ectodomain further

reiterated that, antibodies against conserved regions of the molecule were predominant in majority of the individuals (Cortés *et al.* 2005).

Four conformational epitopes, two by measuring the binding of mutants of the protein to antibodies (Coley *et al.*, 2001), one each by X-ray crystallography (Bai *et al.* 2005) and by incorporating a synthetic peptide into an immunopotentiating reconstituted influenza virosome (IRIV) carrier/adjuvant system (Mueller *et al.* 2003) were identified in AMA-1. Of these, two epitopes identified by Coley *et al.* (2001) and the epitope identified by Mueller *et al.* (2003) was located in the ectodomain of this protein while, the epitope identified by Bai and co-workers, (2005) was placed in the pro sequence. Deeper understanding of candidate molecule structure is imperative to guide more refined vaccine development, especially when the aim is to focus immune responses to conserved, functionally crucial epitopes (Ramarque *et al.* 2007). Such epitopes exist, as evidenced by 4G2, the anti-PfAMA-1 invasion-inhibitory monoclonal antibody (Chesne-Seck *et al.* 2005; Pizzaro *et al.* 2005; Collins *et al.* 2007). According to Remarque and colleagues (2007), amino acid region 302-313 corresponding to the PvAMA-1 Sal I strain was considered to be useful for the 4G2 epitope formation. Additionally, this region overlapped with the amino acid sequence of peptide P04 and was conserved among all Sri Lankan clinical isolates. However, that the 4G2 epitope binds to correctly folded AMA-1 but not to the reduced or alkylated antigen demonstrates that the epitope is conformational and not linear (Kocken *et al.* 1998). Therefore, the elevated anti-PvAMA-1 antibody responses against P04 may be strictly towards a dominant linear B cell epitope present in this region.

Strikingly, as has been reported previously to the recombinant PvAMA-1 protein (Wickramarachchi *et al.* 2006), in the present study, the reduction of IgM prevalence against P04 with increasing exposure to malaria among residents from endemic areas was evident with concurrent increase in IgG prevalence. Thus, under low transmission, it was evident that with increasing exposure to malaria, the bias of the anti-PvAMA-1 antibody response against P04 was towards the IgG isotype, both at population and at individual levels. The switch to IgG anti-PvAMA-1 antibody responses in repeatedly and frequently exposed individuals from the endemic areas may point to a “protective” nature of these antibodies against *P. vivax* infections. Moreover, protective IgG antibodies present in malaria immune adults are only confined to limit the parasites, and not entirely to clear parasitaemia. Therefore, the protective immune response was hypothesized to be dependent on the presence of small sub-clinical

levels of parasites (Pombo *et al.* 2002). Indeed, such naturally acquired immunity is lost if the subject moves out of endemic areas, thus losing the exposure to the pathogen. However, though majority of patients from malaria free Colombo, were adults returning from visits to regions with *P. vivax* transmission (Fonseka and Mendis, 1987), they recorded very low anti-IgM antibody prevalence to both P04 and P05.

Though the predominant isotype in the endemic areas is IgG (Braga *et al.* 2006; Wickramarachchi *et al.* 2006 & 2007), Anuradhapura documented a high prevalence of anti-IgM antibody prevalence against P05. However, the prevalence of IgM antibody responders against P05 decreased with increased exposure, where majority of individuals were exposed to the disease 0 – 4 times. Previous studies documented that, IgM antibodies tend to recognise and bind directly to trophozoite or schizont infected RBC's. Although displaying lower affinity for antigen than IgG isotypes, IgM displayed increased avidity due to its pentameric structure that led to neutralization and agglutination of merozoites and parasitized RBC's (Nebie *et al.* 2008).

Domain II of PvAMA-1 consists of 4 cysteine residues that form disulfide bonds which are important for the folding of this molecule *in vivo*. Of the four cysteine residues, two were located in the peptide sequence of P06, which is suggestive of the inclusion of conformational epitopes. Therefore, the low antibody responses directed against P06 is plausible as the synthetic peptides contain linear B cell epitopes. Structural analyses of domains II and III further confirmed that the disulfide bonds in these individually expressed and refolded domains are correct (Reviewed in Cortés *et al.* 2005).

Udhayakumar and co-workers, (2001) demonstrated that serum antibody responses to B cell epitopes reached a peak prevalence and magnitude between the ages 5–9 years for certain epitopes, whereas, some epitopes generate peak prevalence of responses in older children, and other epitopes only in adults. Therefore, the immune response against malarial antigens continuously evolves over the course of several years, and may explain why complete acquisition of immune responses to a protective repertoire of antigen takes several years of exposure to parasites. This could explain the elevated positive trend observed in individuals from Kataragama P01, P02, P04, P05 and P07 and the positive correlation between the antibody magnitudes against P05 of individuals from Anuradhapura with increasing exposure. Though, a positive correlation was observed between antibody magnitudes against P06 and age, the number of samples were limited (N=9). Further, Mendis *et al.* (1990 & 1992) has

stated that such an age correlation is very unlikely to exist, due to low inoculation rates and heterogeneous exposure among different age groups in Sri Lanka. While, a positive correlation between parasitaemia and the total antibody responses against P03 from Kataragama signified control of the disease, a negative correlation between parasitemia and the IgG antibody responses against P05 from Anuradhapura does indeed indicate the protective nature of parasite specific IgG antibodies.

Among the 21 amino acid haplotypes identified at the PvAMA-1 domain II, haplotype H1 was the predominant haplotype prevalent in Sri Lanka. Further this haplotype which was identical to the PvAMA-1 domain II Sal I strain included 04, 01 and 06 clinical isolates from Colombo, Anuradhapura and Kataragama, respectively. Of the 11 clinical isolates, 2 lacked antibody data while rest of the 9 homologous serum samples responded differentially to the seven synthetic peptides. However, 89% of individuals recorded total (IgM + IgG) antibodies against P04 and P05. As, individuals living in hyperendemic regions and areas where malaria transmission is seasonal are often infected with multiple parasite clones, the inability to rapidly identify the predominant parasite clone in mixed infections is an important limitation because this clone is likely to be most responsible for the disease (Cui *et al.* 2003; Cole-Tobian *et al.* 2007). Typically, a clinical case of malaria arises from infection by a strain to which the host has little immunity or to a strain with greater virulence, which is also likely to be the most abundant clone (Kun *et al.* 2002). The ability to determine the relative abundance of different parasite strains is also important after vaccination for malaria. Most malaria vaccines represent proteins or peptides corresponding to the most abundant allele(s). In vaccination trials, immunization may result in preferential reduction in parasites corresponding to the homologous allele, but vaccination may be less efficacious against reduction for parasites expressing heterologous alleles (Genton *et al.* 2002; Fluck *et al.* 2004).

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 (Anders *et al.* 2004). 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 (Hodder *et al.* 2001; Kocken *et al.* 2002; Healer *et al.* 2004). 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 (Crewther *et al.* 1996). 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 (Doolan *et al.* 2009). Except for peptides P04 and P05, rest of the peptides clearly demonstrated a strain-transcending (cross reactive) immunity in individuals from Sri Lanka, where individuals responded to each peptide irrespective of its diverse amino acid haplotype homology to the PvAMA-1 domain II Sal I sequence.

Though, P04 and P05 was identical to the PvAMA-1 Sal I strain, 18% and 19.6% of individuals lacked antibodies against these two peptides, respectively. The ability to respond to a particular antigen in part is determined by genetic factors, as the influence of human MHC alleles on immune responses to epitopes of parasite antigens (Johnson *et al.*, 2004). Amino acid region corresponding to P04 and P05 predicted two T cell epitopes of which one was predicted to MHC allele HLA-DRB*0401 and the other to HLA-DRB*0101, 0701 and 1101. The establishment of a host-parasite interaction, such that the host is clinically normal and the parasite is also transmitted, is an equilibrium situation normally favoured in evolution. This state arises when there is a sufficient immune response to control the levels of parasitaemia. It is known that malaria parasites generate immune evasive ‘smoke-screen epitopes’ to keep the host immune response busy, but may not evoke immune responses to control the parasite levels effectively. Nonetheless, an effective control occurs only when immune responses against crucial epitopes are generated, the presence of the parasite appears to limit such immune response (Mannan *et al.* 2003). The positive and negative anti-PvAMA-1 domain II antibody responses to both the P04 and P05 peptides observed in 2 individuals from Colombo, who were exposed to the infection 0 and 1 times, respectively while recording a parasite strain identical to the PvAMA-1 domain II Sal I, may imply “Clonal imprinting” or “original antigenic sin”. This hypothesis suggested that an individual’s B cell repertoire against parasite antigens might become fixed by the individual’s early exposure to a particular parasite antigenic variant, thereby preventing the recognition of other variant antigens in subsequent infections (Riley *et al.*, 1996; Taylor *et al.*, 1996). Further, of the 11 individuals who lacked total antibodies against all seven peptides, 9 responded against the recombinant PvAMA-1, PvMSP-1₄₂ and PvMSP-1₁₉ proteins which may include other contributory factors.

The present study demonstrated the widespread immunogenicity of the PvAMA-1 domain II derived synthetic peptides in malaria exposed individuals in Sri Lanka. Bharadwaj et al. (1998) suggested that, through the use of synthetic peptides it may be possible to focus antibody responses on the epitopes which tend to remain cryptic during immunization with the intact protein. However it should also be emphasized that it is not always possible to predict the nature of immune responses from multiepitopic peptides. Further, such immunogens may be polar, and no rules have yet been designed to these molecules for specific immune responses (Bharadwaj *et al.*, 1998). Along with the above initiative, future experiments should be carried out to detect the T cell responses against these seven peptides (Joshi *et al.*, 2000), as evidence can be gained on how these peptides with no apparent sequence homology can cross react both at the T and B cell levels. This will be useful for vaccine target antigens as it is essential to identify B and T cell epitopes that elicits robust responses to develop a subunit vaccine construct.

As for the domain II of PvAMA-1, the naturally acquired antibodies against different PvAMA-1 domain II strains recognised the seven PvAMA-1 domain II synthetic peptides resembling the native Salvador I construct. Further, immune responses in the endemic areas developed to both linear and conformational B cell epitopes in PvAMA-1 domain II. Though, individuals displayed a strain-transcending immune response against PvAMA-1 domain II P07, residues that are essential for the 4G2 conformational B cell epitope formation present in peptide P04 clearly elicited an immune response among individuals in all three study areas. Moreover, the protective immune response generated against the PvAMA-1 was apparent by the marked isotype switch from IgM to IgG against P04 with increasing exposure in residents from areas of endemicity.

Chapter 6. Genetic diversity of the region II of *P. vivax* Duffy binding protein (*PvdbpII*)

6.1. Introduction

Erythrocyte invasion by *Plasmodium* merozoites is an energy dependent, active, complex process that involves a number of interactions between parasite ligands and host receptors. While initial attachments involves merozoite surface proteins, the subsequent events of reorientation, junction formation and invasion require the contents release of three intracellular merozoite apical organelles: microneme, rhoptries and dense granules.

Immediately prior to the formation of the tight junction, the merozoite is able to discriminate between different types of erythrocytes, probably through specific receptor ligand interactions (Lyer *et al.* 2007). *P. vivax* merozoites are only able to invade reticulocytes that express Duffy blood group antigen (DA) (also known as Duffy Antigen Receptor for Chemokine – DARC). The recognition of the human DA is mediated by the *P. vivax* Duffy binding protein (PvDBP) (Chitnis *et al.* 1996), which belongs to the Erythrocyte Binding Ligand (EBL) family. The EBL proteins are localized in the micronemes, but later exported to the merozoite surface during invasion, and are coded by the erythrocyte-binding-like (*eb1*) genes, which consist of single or low copy genes (Adams *et al.* 1992). *P. vivax* has a single known *eb1* gene, which encodes for a single protein, the PvDBP (Adams *et al.* 1992). In contrast, *P. falciparum* consist of a repertoire of several EBL proteins that include EBA-175, EBA-140, EBA-181, MAEBL (Triglia *et al.* 2001, Blair *et al.* 2002; Gilberger *et al.* 2003). The EBL protein divided into six regions that include two cysteine-rich domains, regions II (at -NH₂ end) and VI (at -COOH end), that contain cysteine rich adhesion domains, known as the Duffy Binding Like (DBL) domain, mediates the recognition / binding with the appropriate erythrocyte receptor (reviewed by Howell *et al.* 2006).

The receptor binding DBL domain of PvDBP lies in region II (PvDBPII), that contains approximately 330 amino acid (aa) residues including 12 conserved cysteines and numerous aromatic residues (Adams *et al.* 1992; Chitnis and Miller, 1994). The erythrocyte binding residue of PvDBPII lies in a central 170-aa stretch between cysteines 4 and 8 (Ranjan and Chitnis, 1999). According to the proposed Pv/Pkα DBL 3D structure, six aa residues - Try 94, Asn 95, Lys 96, Arg 103, Leu 168 and Ile 175, that lie between cysteines 4 and 6 come together in three dimensional space to form the exact DA recognition site of PvDBPII during

RBC invasion (Singh *et al.* 2006). Several other additional residues found to be dispersedly located on the outside of the proposed binding surface are suggested to be involved in recognition of the host receptor or subsequent molecular changes or interactions that stabilize the receptor ligand complex (reviewed by McHenry and Adams, 2006).

Although extensive sequence polymorphisms in PvDBPII have been found in *P. vivax* field isolates, the contact residues that form the DA recognition site within PvDBPII appear to be invariant. Analysis of sequence-polymorphism data from diverse geographical locations suggests that there are two contiguous stretches of amino acids within PvDBPII, namely, amino acids from 123 to 149 and 247 to 269, which contain clusters of polymorphic residues with more than 40% of residues being polymorphic. These polymorphism clusters lie on the surface opposite to the DA recognition site, suggesting that naturally acquired antibody responses are directed predominantly against regions distal to the proposed recognition site.

The universal dependent on the interaction between PvDBP with DA for erythrocyte invasion and lack of alternative invasion pathways (as in *P. falciparum*) makes PvDBPII one of the leading vaccine candidate antigen against blood stage of *P. vivax* malaria. Two key observations, *i.e.* individuals that lack the expression of the DA on RBC surface are completely resistant to *P. vivax* merozoite invasion (Miller *et al.* 1976), and reduced susceptibility to *P. vivax* infection of heterozygous carriers of a Duffy-negative allele compared with wild-type homozygotes (Kasehagen *et al.* 2007), provide strong evidence that complete or partial disruption of the expression of DA reduces invasion ability and may restrict the blood stage development of *P. vivax*. Dominant T cell and B-cell epitopes have been identified within the receptor binding region of PvDBP and residents of areas endemic for *P. vivax* develop progressively stronger humoral and cellular immune responses following repeated exposure against this antigen (Fraser *et al.* 1997; Michon *et al.* 2000; Xainli *et al.* 2002; Xainli *et al.* 2003). Recent findings showed that anti-PvDBPII antibodies obtained from individuals exposed to *P. vivax*, or artificially induced in rabbits, can partially inhibit *P. vivax* merozoite invasion *in vitro* (Grimberg *et al.* 2007). Further observations that naturally acquired PvDBPII specific binding inhibitory antibodies confer protection against blood stage *in vivo*, provide ample evidence that protective immune response to *P. vivax* is at least partially directed against PvDBP, reiterate the importance of developing a vaccine based on this antigen (King *et al.* 2008). Recent findings that the naturally acquired immunity against PvDBPII contained both strain specific as well as strain transcending (cross reactive immunity) components further validated its candidacy as potential vaccine against blood stage

of *P. vivax* malaria (Cole-Tobian *et al.* 2009). Together with these findings, the conserved nature of the DA binding site and segregation of the polymorphic residues in to two regions located on the opposite face to the DA recognition site of the *PvdbpII* gene among the field isolates collected from different malaria endemic areas world wide, suggest that anti-PvDBP II binding inhibitory antibodies should be able to block the binding of polymorphic receptor binding domains of PvDBP derived from diverse *P. vivax* field isolates (reviewed in Chitnis and Sharma, 2008). A vaccine based on PvDBP II would thus be expected to be effective against diverse *P. vivax* isolates. PvDBP II plays an important role in determining the efficacy of the naturally acquired protective immune responses contained both strain specific as well as strain transcending immunity and the level of genetic diversity directly associated with malaria transmission intensity.

6.2 Objectives

1. To characterize the prevailing genetic diversity associated with the 224 amino acid fragment containing the critical binding motif of the region II of the *Pvdbp* gene among *P. vivax* field isolates
2. To examine the specific functionality of naturally acquired anti-DBP antibodies in terms of binding inhibition of recombinant PvP II to recombinant Duffy antigen.
3. Evaluation of the ensuing strain specific antibody mediated immunity to *P. vivax* DBP.

6.3. Results

Hundred *P. vivax* isolates (those confirmed as single clon infections) were used to investigate the genetic diversity of *PvdbpII* gene from two malaria endemic and a single non endemic sites in Sri Lanka. 224 amino acids (spanning from 46 to 269) including the critical binding motif (CBF) (cysteines 4-8; approximately 170 amino acids) and two areas where the polymorphic amino acids clustered (Singh *et al.* 2003; Singh *et al.* 2006) were also included in this portion of the *PvdbpII* gene (Figure 6.1). The complete sequences of 100 Sri Lankan isolates were submitted to the GenBank (<http://www.ncbi.nlm.nih.gov>) under the accession numbers GU143914 - GU144013. Parasite isolates collected from the malaria endemic areas (EA), Anuradhapura (N = 24) [GU143950 – GU143973] and Kataragama (N = 40) [GU143974 – GU144013] and from malaria non-endemic area Colombo (N = 36) [GU143914 - GU143949].

Patients from non endemic Colombo had been infected with malaria disease during visits the regions of the island with natural *P. vivax* transmission occurred in the country. According to the information provided by these patients, the different areas of the country, from where those patients acquired the disease were identified. This group contained isolates acquired from 11 different administrative districts of Sri Lanka spanning entire endemic areas of the country. These districts included Ampara (N=1), Batticaloa (N=1), Jaffna (N=1), Kurunegala (N=6), Moneragala (N= 4), Polonnaruwa (N =2), Puttalam (N=3), Ratnapura (N=4) and Vavunia (N=3). Further, 2 patients probably acquired the disease from India.

6.3.1. Sequence polymorphism of the *PvdbpII* gene of Sri Lankan isolates

Sequence analysis of local *P. vivax* isolates revealed that, although size polymorphism was not observed, single nucleotide polymorphisms (SNP's) were present within the sequenced region of the *PvdbpII* gene.

Compared to the sequence of the *PvdbpII* gene of the reference *P. vivax* Salvador-1 (Sal-1) strain, 27 variant nucleotides were found among the Sri Lankan isolates (Table 6.1). All these sites were dimorphic and resulted in the same number of mutations (S=27) at the nucleotide level. From the total 27 mutations recorded, 23 were non synonymous while the rest were synonymous mutations (Table 6.1). Of the 224 amino acids, polymorphisms occurred at 21 sites (9%), compared to the reference Sal-1 strain, where 20 were monomorphic mutations (change into one other amino acid type) and only one position (508) showed a dimorphic mutation (change into 2 other amino acid types).

Nucleotide mutations found among the local isolates occurred at all three bases of the codons (Table 6.1) of the *PvdbpII* gene. Of these mutations, 10 each occurred at the first and third base positions while 6 occurred at the second base position of the codons, which resulted in significant amino acid changes in the PvDBPII protein among the Sri Lankan isolates. Apart from the codon at position 508, in which mutations occurred at all three base positions, other mutations were restricted to only a single base position of a particular codon. All 6 cysteines with certain hydrophobic residues within the amplified gene fragment were

46 M K E L T N L V ³⁰⁰ N N T D T N ³⁰⁶ F H ³⁰⁸ R D I T F R K L Y L K R K L I Y
 78 D A A V E G D L L K L N N Y R Y N K D F C₅ K D I R W S L G D
 109 F G D I I M G T D M E G I G Y S ³⁷¹ K V V E N N L R S I F G T ^{384 385 386} D E K
 141 ³⁹⁰ A Q Q R R K Q W W N E ³⁹⁸ S K A Q I W ⁴⁰⁴ T A M M Y S V K K R L K G
 171 ⁴¹⁷ N F I W ⁴¹⁹ I ⁴²⁴ C₆ K L N V A V N I E P Q I Y R ⁴³⁷ W I R E W G R D Y V S
 202 E L P T E V Q K L K E K C₇ D G K I N Y T D K K V C₈ K V P P C₉ Q
 233 N A C₁₀ K S Y D ⁴⁸⁶ Q ⁴⁸⁹ W I T R K K N Q W D V L S N K F ^{503 504 505} I S V K N A ⁵⁰⁸
 263 ⁵¹³ E K V Q T A G

Figure 6.1: Amplified fragment of the region II of the *P. vivax* Duffy Binding Protein (with 224 amino acids).

Amino acids at the beginning of each row are numbered according to Singh *et al.* 2006, Polymorphic residues are numbered according to Fang *et al.* 1991.

Minimal 170 amino acid Duffy receptor binding region (yellow colour area), two clusters of polymorphic residues (grey areas) (one contained within the critical binding motif), 21 polymorphic residues found among the 100 Sri Lankan sequences (red), conserved cysteines (blue), residues essential for Duffy antigen recognition (squared blue).

invariant among the local isolates. Further, according to the *P. knowlesi* DBPa (*Pkdbpa*) / PvDBP crystal structure (Sing *et al.* 2006), six discontinuous surface exposed amino acid residues described as essential contact residues (Tyr 340, Asn 341, Lys 342, Arg 348, Leu 459 and Ile 466) were also conserved among the 100 isolates analysed in the present study, confirming previous studies (reviewed by Chitnis and Sharma, 2007). However, VanBuskirk *et al.*, (2004 a), described additional residues located between cysteines 4 -7, involved in the DA binding or having a role in receptor recognition. Of these residues, 20 were identified as essential (in which polymorphism of these residues completely abrogates the binding with DA receptor), 22 as less important and 35 as residues that had no effect on binding (VanBuskirk *et al.* 2004 a). Among the Sri Lankan field isolates, from each of the two groups described as critical and less important, a single polymorphic residue was recorded (F306L from the essential group and W437R from the less important groups).

Of the 21 polymorphic sites recorded in this study, 13 (62%) were found within the minimal critical binding motif (CBM) of the PvDBPII ligand domain. According to the deduced crystal structure, a majority of the polymorphic amino acid residues were clustered in two areas of the PvDBPII molecule, located opposite to the Duffy receptor recognition site (Singh *et al.* 2006). However, of the 21 polymorphic residues in Sri Lankan sequences, only 48% were found within these two polymorphic areas (Figure 6.1). Other polymorphic sites, which remain as isolated residues, were distributed throughout the molecule and some of those point polymorphisms also lie close to the portion of the ligand domain critical for DA receptor recognition.

Polymorphisms N417K, W437R and I503K, either in single or in combination, can alter the efficacy of an acquired binding inhibitory antibody response (VanBuskirk *et al.* 2004a). Locally, variants N417K, W437R and I503K were found at frequencies of 36%, 37% and 55%, respectively. Analyses of the combination of variants revealed that N417K-W437R occurred at a high frequency (99%), while N417K-I503K and W437R-I503K occurred at frequencies of 27% and 30%, respectively. This suggests that though a strong association occur between N417K-W437R, no such strong association was found between I503K with either N417K or W437R, among local PvDBPII alleles.

Different arrangements of the nucleotide polymorphisms resulted in 39 different nucleotide haplotypes (h=39). Most of the Sri Lankan isolates belonged to three different haplotypes, representing 18%, 15% and 14% of the total isolates. Of the 39 haplotypes, only two were

distributed in all test areas, while another 29 different haplotypes were restricted to a single test area (10 from Kataragama and 9 each from Anuradhapura and Colombo). Further, only one contained identical nucleotide sequences to the reference strain Sal-1.

The distribution of the polymorphic amino acids in to different patterns resulted in 33 PvDBPII amino acid haplotypes / strains among the Sri Lankan isolates (Table 6.2). More than 50% of the total isolates grouped in to three different amino acid haplotypes (SL 1, 2 & 3) which comprised 21%, 16% and 15% isolates respectively, while another 21 were restricted to single isolates each. Distribution of the most prevalent PvDBPII haplotypes within the country (SL 1 in 7 districts, SL 2 and SL 3 in 4 districts) revealed that no significant isolation of alleles occur with respect to different districts among these alleles within the country. Of the rest, 6 were distributed in two areas while another 24 were restricted only to a single test area each. Further, the two sequences of the two isolates collected from patients who acquired the diseases from South India were identical to SL 1 and SL 2 types, the most frequent allele types in Sri Lanka.

Table 6.1: Nucleotide and amino acid changes compared to the reference Sal-1 sequence within the 672 bp region of the *PvdbpII* gene among the Sri Lankan isolates

[¥] Position ^a	300	306	308	371	378	384	385	386	390	398	404	417	419	424
Position ^b	54	60	62	125	132	138	139	140	144	152	158	171	173	178
*														
Sal-1	AAT	TTT	AGG	AAA	CGC	GAT	GAA	AAG	CGT	TCT	ACA	AAT	ATA	TTA
Sri Lanka	<u>TAT</u>	<u>TTG</u>	<u>AGT</u>	<u>GAA</u>	<u>CGT</u>	<u>GGT</u>	<u>AAA</u>	<u>AAT</u>	<u>CAT</u>	<u>ACT</u>	<u>AGA</u>	<u>AAA</u>	<u>ATG</u>	<u>ATA</u>
Sal-1	N	F	R	K	R	D	E	K	R	S	T	N	I	L
Sri Lanka	Y	L	S	E	R	G	K	N	H	T	R	K	M	I
Percentage ^c	3%	7%	13%	34%		94%	20%	20%	66%	12%	12%	36%	18%	49%

Position ^a	437	465	476	486	489	503	504	505		508 [#]		513	515
Position ^b	191	219	230	240	243	257	258	259		262		267	269
		*	*										*
Sal-1	TGG	ATC	CCA	CAA	ACC	ATA	ATG	GTA		GCA		ACG	GCA
Sri Lanka	<u>CGG</u>	<u>ATA</u>	<u>CCC</u>	<u>GAA</u>	<u>GCC</u>	<u>AAA</u>	<u>AGG</u>	<u>TTA</u>	<u>CGA</u> / <u>CGC</u> / <u>CCA</u>		<u>AAG</u>	<u>GCC</u>	
Sal-1	W	I	P	Q	T	I	S	V		A		T	A
Sri Lanka	R	I	P	E	A	K	R	L		R / R / P		K	A
Percentage ^c	37%			1%	3%	55%	3%	3%		11% / 1% / 1%		24%	

[¥]Number of the amino acid residue - ^aAccording to Fang *et al.* 1991; ^bAccording to Sing *et al.* 2006
(bold and underlined nucleotides are the variants compared to reference Sal-1 sequence, within that particular codon).

^c Percentage of detection frequency of amino acid in the Sri Lankan isolates

* Positions of synonymous nucleotide changes; [#] Dimorphic mutation (changed in to two amino acid types)

Table 6.2: PvDBPII amino acid haplotypes recorded among the Sri Lankan *P. vivax* field isolates

Strain	Amino acid position																				% #	Area			Different administrative districts in the country *	
	300	306	308	371	384	385	386	390	398	404	417	419	424	437	486	489	503	504	505	508		513	C	A		K
Sal-1	N	F	R	K	D	E	K	R	S	T	N	I	L	W	Q	T	I	S	V	A	T					
SL 1	.	.	.	.	G	.	.	H	.	.	.	.	.	.	.	.	K	.	.	.	.	21	9	2	10	Ku, Am, Mo, Pu, Ha, In
SL 2	.	.	.	E	G	.	.	.	.	.	K	M	I	R	.	.	.	.	.	.	.	16	7	5	4	Va, Ku, In
SL 3	.	.	.	.	G	.	.	H	.	.	.	.	.	.	.	.	K	.	.	.	K	15	2	1	12	Pu, Ba
SL 4	.	.	.	E	G	K	N	H	.	.	K	.	I	R	.	.	.	.	.	.	.	6	2		4	Mo, Ja
SL 5	.	L	S	.	G	K	N	H	T	R	.	.	I	.	.	.	.	.	.	.	.	5	2	3		Ku
SL 6	.	.	.	.	G	.	.	H	.	.	.	.	.	.	.	.	K	.	.	R	.	4	3		1	Mo, Ra
SL 7	.	.	.	E	G	.	.	.	.	.	K	.	I	R	.	.	K	.	.	.	.	3	1	2		Ap
SL 8	.	.	S	.	G	K	N	H	T	R	.	.	I	.	.	.	.	.	.	.	.	3	1	2		
SL 9	.	.	.	.	G	.	.	H	.	.	.	.	.	.	.	.	K	.	.	R	K	2		2		
SL 10	.	.	.	.	G	.	.	H	.	.	.	.	.	.	.	.	.	.	.	.	.	2	1			Mo
SL 11	.	.	S	.	G	K	N	H	T	R	.	.	I	.	E	.	.	.	.	.	.	1			1	
SL 12	.	L	S	.	G	K	N	H	T	R	.	.	I	.	.	.	.	R	L	.	K	1		1		
SL 13	Y	L	S	.	G	K	N	H	T	R	.	.	I	.	.	.	.	.	.	.	.	1		1		
SL 14	.	.	.	.	.	.	.	.	.	.	K	.	I	R	.	.	K	.	.	.	.	1	1			Po
SL 15	.	.	.	.	G	.	.	H	.	.	.	.	.	.	.	A	.	.	.	.	.	1			1	
SL 16	.	.	.	E	G	.	.	.	.	.	K	M	I	R	.	.	.	.	.	R	.	1	1			Ra
SL 17	Y	.	.	.	G	.	.	H	.	.	.	.	.	.	.	.	K	.	.	.	.	1	1			Ap
SL 18	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	1			1	
SL 19	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	L	.	.	1		1		
SL 20	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	.	1			1	
SL 21	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	K	1	1			Ra
SL 22	.	.	.	E	G	.	.	.	.	.	.	.	I	R	.	.	K	.	.	.	.	1	1			Po
SL 23	.	.	.	.	.	.	.	.	.	.	K	.	I	R	.	.	K	R	L	.	.	1		1		
SL 24	.	.	.	.	G	.	.	.	.	.	K	.	I	R	.	.	K	.	.	.	.	1		1		
SL 25	Y	.	.	E	G	.	.	.	.	.	K	.	I	R	.	A	K	.	.	R	.	1			1	
SL 26	.	.	.	E	G	.	.	.	.	.	K	.	I	R	.	A	K	.	.	R	K	1		1		
SL 27	.	.	.	E	G	.	.	.	.	.	K	.	I	R	.	.	K	.	.	R	.	1			1	
SL 28	.	.	.	.	G	.	.	H	.	.	.	.	.	.	.	.	K	.	.	P	K	1			1	
SL 29	.	.	S	.	G	K	N	H	T	R	.	.	I	.	.	.	.	.	.	.	K	1	1			
SL 30	.	.	.	E	G	K	N	H	.	.	K	.	I	R	.	.	.	.	.	.	K	1	1			Ku
SL 31	.	.	S	E	G	K	N	.	.	.	K	.	I	R	.	.	K	.	.	.	.	1	1			Ra
SL 32	.	.	.	E	G	.	.	.	.	.	K	.	I	R	.	.	.	.	.	.	.	1			1	
SL 33	.	.	.	E	G	.	.	.	.	.	K	M	I	R	.	.	.	.	.	.	K	1		1		

Percentage of isolates observed

*Am = Ampara; Ap = Anuradhapura; Ba = Badulla; Ha = Hambantota; Ja = Jaffna; K = Kalutara; Mo = Moneragala; Ku = Kurunegala; Pu = Puttalam; In = India; Va = Vaunia; Ra = Ratnapura; Po = Pollinnaruwa

6.3.2. Comparison of PvDBP II Sri Lankan sequences with worldwide sequences

Hundred sequences obtained from the present study were aligned and compared with the previously published sequences data from malaria endemic areas in diverse geographic regions; Colombia (N =17), Papua New Guinea (N=111), South Korea (N=13) and Thailand (N =30).

All cysteines and the six amino acid residues that were predicted to be directly involved in DA receptor recognition were conserved among the global isolates. However of the 271 sequences, polymorphisms were recorded at 83 positions compared to the laboratory reference Sal-1 sequence (Table 6.3). Among these polymorphic sites, only 10 were dimorphic while the rest were monomorphic. Of these, 31 (37%) variable amino acids were located within the two areas where polymorphic residues were clustered as described in the PvDBL crystal structure (Singh *et al.* 2006).

Of the 21 polymorphic amino acids found in local isolates, 18 with exact amino acid variants (306, 308, 371, 384, 385, 386, 390, 398, 404, 417, 419, 424, 437, 486, 503, 504, 505 and 513), were previously recorded either in single or few isolates from different malaria endemic areas globally (Table 3.4.3). Of these 16 (76%) were previously recorded from Thailand, 10 (48%) from PNG and South Korea, and 9 (43%) from Colombia. In comparison, three polymorphisms found within the Sri Lankan isolates - N300Y, T489A and A508R / A508P, unique to the country and recorded for the first time (Table 3.4.3). Polymorphisms at 8 different sites recorded as the most common variant sites in PvDBPII among globally, compared to the Sal-1 strain (Table 6.4) (Sousa *et al.* 2005; Gosi *et al.* 2007). Of these, apart from S447K unique to Papua New Guinea and K386Q recorded in Thailand and Papua New Guinea, all other variants were found in local isolates, also at high frequencies.

The distribution of the variant amino acids in to different patterns resulted in 117 different haplotypes of the *PvdbpII* globally. Of these the haplotype with highest frequency (N=29), which was identical to the Sal-1 sequence, found only in Papua New Guinea (N=28) and Sri Lanka (N=1). Apart from this, 32 other haplotypes were found among the local isolates. Some of these haplotypes were previously recorded in other malaria endemic geographical areas too. Seven of the Sri Lankan haplotypes were recorded in Thailand and one each from Papua New Guinea and South Korea. Interestingly none of the partial variant families found in Sri

Lanka were recorded in Colombia. Further, 25 of the variant families found in this study, were not previously recorded from any other geographical area of the world, and thus seem to be unique to Sri Lanka.

Table 6.3: Polymorphic amino acid sites of the *PvdbpII* gene among isolates collected from different malaria endemic geographical areas

world wide

Amino acid	2	2	2	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	
	9	9	9	0	0	0	0	0	1	1	2	2	3	3	4	4	4	5	6	6	6	6	6	7	7	7	7	7	7	
	3	4	5	0	1	3	6	8	8	9	0	8	3	5	2	8	9	1	2	4	7	8	9	1	2	3	4	5	6	7
Sal-1 [#]	K	E	L	N	N	D	F	R	K	R	K	E	L	L	K	I	R	S	T	M	I	G	Y	K	V	V	E	N	N	L
COL ¹	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	N	E	.	.	.	.	K	.
																							29	24					29	
PNG ²	.	D	.	.	D	.	.	S	R	K	R	.	F	.	E	K	.	.	.	.	.	.	.	E	.	A	.	D	.	W/G/S
		1			1			64	1	1	1		8		1	1								1		1		8		3/2/1
SK ³	S	D	H	.	.	Y	.	.	.	.	.	K	.	S	.	.	T	.	A	L	.	R	.	E/N	.	.	Q	.	.	.
	8	8	8			8						8		8			8		8	8		8		54/8			15			
Thai ⁴	.	.	.	.	.		L	S	.	.	.	.	F	.	.	.	.	C	.	.	T	.	.	E	.	.	.	D	.	.
							6.7	27					47					3.3			6.7			20				7		
SL ⁵	.	.	.	<u>Y</u>	.	.	L	S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	E	.	.	.	.	.	.
				<u>3</u>			7	13																34						

Amino acid	3	3	3	3	3	3	3	3	3	3	3	3	3	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	7	8	8	8	8	8	8	8	9	9	9	9	9	9	0	0	1	1	1	1	1	2	2	2	3	3	3	3	3
	9	0	1	4	5	6	8	9	0	1	3	4	5	6	8	4	7	2	5	7	9	4	8	9	3	4	6	7	9
Sal-1	S	I	F	D	E	K	Q	Q	R	R	Q	W	W	N	S	T	M	K	K	N	I	L	V	N	Q	I	R	W	R
COL	.	.	.	G	K	N	.	.	.	.	.	.	.	.	.	.	.	.	.	K	.	I	I	.	.	.	.	R	.
				53	24	24														53		47	24					12	
PNG	N	.	S	G	K	Q/N	K	H	H	H	R	R	C	S	.	.	.	E	E	K	.	I	.	S	.	T	.	R	L
	1		4	57	8	10/8	1	1	51	1	1	13	2	1				1	1	23		62		1		3		23	1
SK	.	V	.	G	K	N	.	.	H	P	P	L	L	.	.	.	.	.	.	K	.	I	.	.	.	.	.	R	.
		15		54	15	15			54	8	8	8	8							92		100						100	
THAI	.	.	.	G	K	N/Q	.	.	H	.	.	.	.	.	T	R	.	.	.	K	M	I	.	.	K	.	T	R	.
				77	47	40/3			57						10	10				37	3.3	83			3.3		3.3	63	
SL	.	.	.	G	K	N	.	.	H	.	.	.	.	.	T	R	.	.	.	K	M	I	.	.	.	.	.	R	.
				94	20	20			66						12	12				36	18	49						37	

Amino acid	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	5	5	5	5	5	5	5	5	5
	3	4	4	4	5	5	5	5	6	7	8	8	8	8	9	9	0	0	0	0	0	1	1	1
	9	4	5	7	1	2	4	9	7	5	5	6	8	9	2	4	0	3	4	5	7	8	1	3
Sal-1 [#]	R	D	Y	S	T	E	Q	K	T	P	D	Q	I	T	K	Q	N	I	S	V	N	A	V	T
COL	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	K	.	L	.	.	L	.
																		18		35			3	
PNG	L	G	.	K	S	G	K	E	P	.	H	.	V	.	E	R	D/S	K	.	.	.	.	.	.
	1	1		40	1	1	1	1	5		1		1		1	1	1/1	27						
SK	.	.	S	.	.	.	P	.	.	.	.	.	.	.	.	.	.	K	R	.	.	.	.	.
			8				8											100	8					
Thai	.	.	.	.	.	.	.	.	.	A	.	E	.	.	.	.	.	K	.	.	H	.	.	K
										10		6.7						57			3.3			3.3
SL	.	.	.	.	.	.	.	.	.	.	.	E	.	A	.	.	.	K	R	L	.	R/P	.	K
												1		3				55	3	3		12/1		24

Amino acids were numbered according to Fang *et al.* 1991

Sal-1 = *P. vivax* Salvador - 1. (Accession Number = M37514)

* Accession numbers of the isolates

¹COL = Colombia (N = 17) (U50575-50591)

²PNG = Papua New Guinea (N = 111) (AF 469515-469602, AF289480-289483, AF291096, AF289635-289639, AF289641-289653)

³SK = South Korea (N = 13) (AF215737-215738, AF220657-220667)

⁴Thai = Thailand (N = 30) (EF219451, EF368159-368177, EF368179-368180, EF379127-379132, EF379134-379135)

⁵SL = Sri Lanka (N=100) (GU143914 - GU144013)

Table 6.4: Frequencies of the most commonly variant amino acids in PvDBPII in different malaria endemic geographical areas, compared with the Sal-1 sequence

	R308S ^a	D384G	K386N	K386Q	N417K	L424I	W437R	S447K	I503K
Braz	12.5	85	12.5	0	27.5	32.5	27.5	0	55
Col	0	59	23	0	47	47	18	0	12
PNG	67	66	8	11	23	34	26	59	29
SK	0	64	5	0	98	100	100	0	100
Thai	26	76	40	3	36	86	63	0	56
SL	13	94	20	0	36	49	37	0	55

^aThe first letter represents amino acid position in the Sal-1 sequence and the latter the substituting amino acid. (Amino acids were numbered according to Fang *et al.* 1991)

Braz = Brazil; Col = Colombia; PNG = Papua New Guinea; SK = South Korea; Thai=Thai;
SL = Sri Lanka

6.3.3. Test for diversity

Several diversity parameters were considered to determine the diversity of the PvDBPII gene of Sri Lankan isolates. The indicators used, pair wise nucleotide diversity (both in terms of π and d), the number of polymorphic sites (S), the number of different haplotypes (h) with haplotype diversity (Hd) and the number of variant positions (VP) For these variants, the number of singleton positions (SP) and the of Parsimony Informative sites (PI) were considered. Amino acid sequence diversity was estimated with p-distance (with pair wise deletion).

Pair wise nucleotide diversity (π) for 100 Sri Lankan isolates was found to be 0.00982 (Table 6.5). Using Tamura's three parameter model, this value (d) was recorded nearly equal to π ($d = 0.00983$). Between the three test areas, isolates from the Anuradhapura had the highest values for both these parameters ($\pi = 0.0122$ & $d = 0.0123$), followed by Colombo and Kataragama (Table 6.5). Analysis of the distribution of the genetic diversity across the *PvdbpII* gene using the sliding window method showed that values for the π for the 100 sequences ranged from 0 to 0.02094, and the maximum diversity was found between cysteins 5 and 7, while the valleys of low diversity occurred around cystins 5, 6 and 7.

Within the region of the *PvdbpII* gene analysed, 27 polymorphic (segregating) sites were identified among 100 Sri Lankan isolates. The number of polymorphic sites were equal in the two endemic areas (S=24 each) and higher than that of the non-endemic area (S=20) (Table 6.5). Further, among the 100 isolates, 39 different nucleotide haplotypes (h=39) were identified, with haplotype diversity (Hd) 0.922 (± 0.014). Among three test areas, the number of different haplotypes was highest in Colombo (h=19) followed by Kataragama and Anuradhapura. However, the haplotype diversity was highest among isolates from Anuradhapura (Hd = 0.942 { ± 0.031 }) and lowest in Kataragama (Hd = 0.868 { ± 0.035 }).

Three of the polymorphisms (at positions 583, 651 and 669) observed in Sri Lankan isolates were SP variables (found only in a single isolate) while the rest (N = 24) were PI sites (Table 6.5). Papua New Guinean (~10%) and South Korean (~5%) isolates showed highest proportion of variant sites with higher number of SP than PI positions within the analyzed portion of the PvDBPII. Isolates from Sri Lanka, Colombia and Thailand showed higher number of PI sites than SI.

Table 6.5: Indices of the genetic diversity of the 672 bp portion of the *PvdbpII* gene from isolates from different malaria endemic areas worldwide

Locality	N ^a	S ^b	π (SD) ^c	H ^d Hd (SD) ^e	SP ^f	Pi ^g	P(SE) ^h
Sri Lanka (total)	100	27	0.0098 (± 0.0005)	39 0.922 (± 0.014)	3	24	0.025 (0.006)
Colombo ¹ (non-endemic)	36	21	0.0099 (± 0.0008)	19 0.913 (± 0.033)	1	20	0.025 (0.006)
Anuradhapura ² (endemic)	24	24	0.0123 (± 0.0008)	15 0.942 (± 0.031)	4	20	0.031 (0.007)
Kataragama ³ (endemic)	40	24	0.0075 (± 0.0009)	16 0.868 (± 0.035)	8	16	0.019 (0.005)
Papua New Guinea ⁴	111	69	0.0101 (± 0.0005)	56 0.936 (± 0.015)	42	27	0.024 (0.005)
Colombia ⁵	17	14	0.0090 (± 0.0007)	15 0.985 (± 0.025)	0	14	0.027 (0.007)
South Korea ⁶	13	33	0.0105 (± 0.0033)	10 0.962 (± 0.041)	26	7	0.026 (0.006)
Thailand ⁷	30	29	0.0112 (± 0.0005)	24 0.983 (± 0.015)	11	18	0.029 (0.007)

^a Number of sequences sampled; ^b Number of polymorphic (segregating) sites; ^c Pairwise nucleotide diversity (standard deviation); ^d Number of nucleotide haplotypes; ^e Haplotype diversity (standard deviation); ^f Number of singleton variables; ^g Number of parsimony informative sites; ^h Pairwise amino acid diversity (standard error);

Accession numbers:

1 = GU143914-GU143949

2 = GU143950 – GU143973

3 = GU143974 – GU143913

4 = AF469515-469602, AF289480-289483, AF291096, AF289 635-289639, AF289641-289653

5 = U50575-50591

6 = AF215737-215738, AF220657-220668

7 = EF219451, EF368159-368177, EF368180, EF379127-379132, EF379134-379135

6.3.4. Test of neutrality and natural selection

Four tests of natural selection were performed. The average number of synonymous (dS) and non-synonymous (dN) substitutions, the McDonald-Kreitman test, Tajima's D-test and Fu and Li's *D* and *T* tests.

The dN - dS was >1 for all 100 isolates and for isolates from each test area, suggesting that this part of the PvDBP protein was mostly subjected to directional or diversifying selection (Table 6.6). However, the Z-test for all isolates as well as isolates from the three test areas separately did not show evidence for departure from neutrality. For all cases, Z-test did not result in significant values for the probability of rejecting the null hypothesis of strict neutrality (dN = dS) in favour of alternative hypothesis of positive selection (dN > dS).

In McDonald-Kreitman test (McDonald and Kreitman, 1991) 03 published *P. knowlesi dbp-α* gene (L14805-L14807) sequences used as an out group. Fisher's exact test was applied to the data to test for significant non randomness ($P < 0.05$), and the skew from randomness was calculated as the neutrality index. The 100 sequences as a whole showed high excess of dN polymorphisms ($P = 0.0465$ based on Fisher's exact test) within *P. vivax* sequences relative to dN polymorphisms from the out group species, *P. knowlesi* (Table 6.6). This indicates that a significant departure from neutrality and suggested purifying selection occurred in this region of the gene operating to reduce the number of dN substitutions between species. Although the dN polymorphisms within *P. vivax* sequences was comparatively higher than that of the out group in the three test areas, it was statistically significant only in Anuradhapura.

In Tajima's D-test the departure from neutrality was measured as the difference between π (observed average pairwise nucleotide diversity) and θ (expected nucleotide diversity under neutrality derived from number of segregating sites). The Fu and Li tests identify departures from neutral patterns of nucleotide substitutions as deviations between the estimates of θ derived from the number of phylogenies compared to either the total number of mutations (*D*) or the average pairwise diversity (*F*). For 100 local isolates, both Tajima's D and Fu and Li's *D* and *F* resulted statistically non-significant positive values (Tajima's $D = 0.7643$ [$P > 0.1$]; Fu and Li's $D = 0.8143$ [$P > 0.1$] and $F = 0.9516$) (Table 6.6). In area wise analysis, non significant positive values were recorded for isolates from malaria non endemic Colombo and endemic Anuradhapura while in Kataragama a non significant negative value resulted for both Tajima's D and Fu and Li's *D* and *F* statistics.

Table 6.6: Selection pressure of the *PvdbpII* gene of the field parasite isolates collected from Sri Lanka

	Rates of synonymous and non synonymous mutations				McDonald-Kreitman test			Tajimas test	Fu and Li's test	
	dN^a	dS^b	$dN-dS^c$ ($\pm SE$)	Z test	(S/Ns) ^d	(S/NS) ^e	Neutrality index (P^f)	Tajimas D	D*	F*
Total	0.0108 (0.003)	0.0058 (0.003)	0.005 (0.004)	$dN > dS$ $P = 0.112$	46 / 71	4 / 20	3.2390 (0.0264*)	0.7643 $P > 0.10$	0.8144 $P > 0.10$	0.9517 $P > 0.10$
Colombo (NEA*), (N=36)	0.0110 (0.003)	0.0057 (0.003)	0.0052 (0.004)	$dN > dS$ $P = 0.111$	46 / 72	3 / 18	3.8330 (0.0288*)	1.0218 $P > 0.10$	1.3458 $0.10 > P > 0.05$	1.4624 $0.10 > P > 0.05$
Anuradhapura (EA*), (N=24)	0.0133 (0.003)	0.0087 (0.005)	0.0046 (0.006)	$dN > dS$ $P = 0.221$	46 / 72	3 / 21	4.4720 (0.0171*)	1.0222 $P > 0.10$	0.6632 $P > 0.10$	0.9050 $P > 0.10$
Kataragama (EA), (N=40)	0.0085 (0.002)	0.0039 (0.002)	0.0046 (0.003)	$dN > dS$ $P = 0.070$	46 / 71	4 / 17	2.7540 (0.0881)	-0.3748 $P > 0.10$	-0.6583 $P > 0.10$	-0.6658 $P > 0.10$

^a Number of nonsynonymous mutations per nonsynonymous site using Nei-Gojobari with Jukes-Cantor correction; ^b Number of synonymous mutations per synonymous site using Nei-Gojobari with Jukes-Cantor correction; ^c Rate of nonsynonymous mutation minus rate of synonymous mutation; ^d Fixed difference between species (synonymous / nonsynonymous); ^e Fixed difference between species (synonymous / nonsynonymous); ^f P value for Fisher's exact test

*NEA = Malaria non endemic area; EA = Malaria endemic area

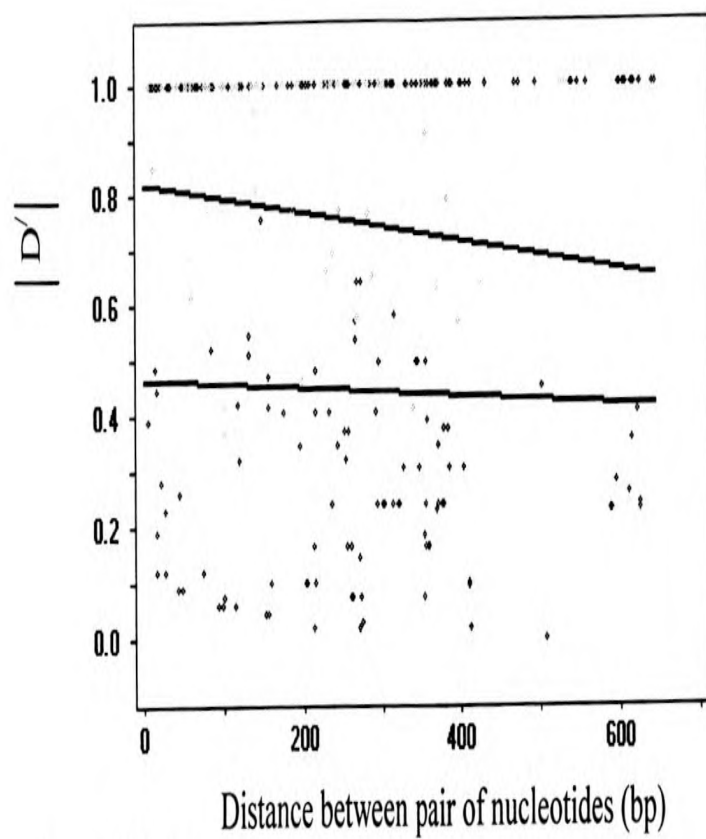
6.3.5. Recombination and linkage disequilibrium

The four gamete test suggests the occurrence of at least 9 recombination events (25 - 45) (51 - 238) (238 - 280) (285 - 296) (296 - 378) (436 - 552) (592 - 635) (635 - 639) (650 - 665), amongst the 100 sequences analyzed. The recombination parameter, *C*, was estimated at 0.0206 between adjacent nucleotides and 13.8 across the whole gene sequence.

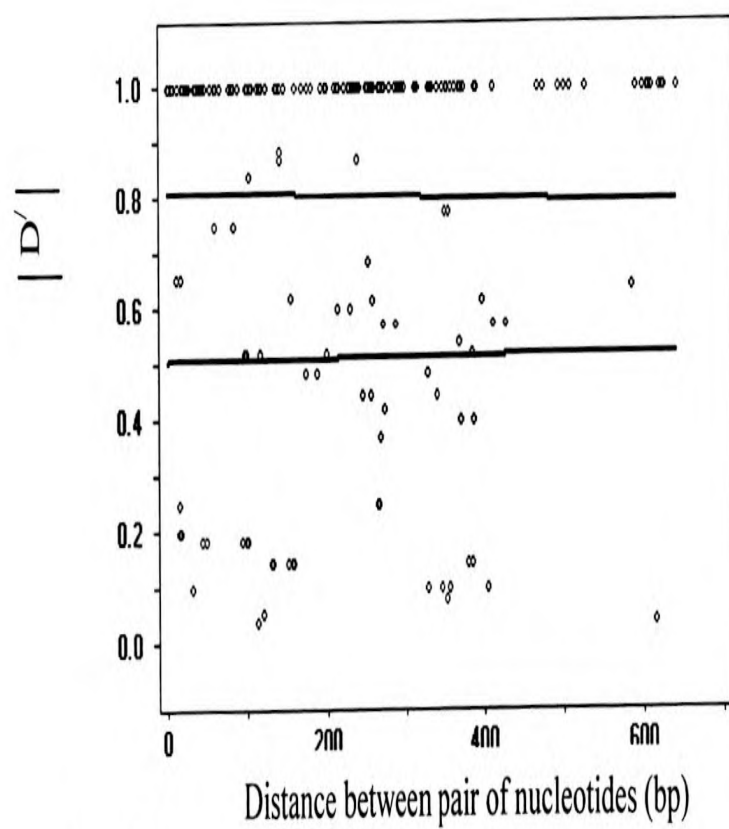
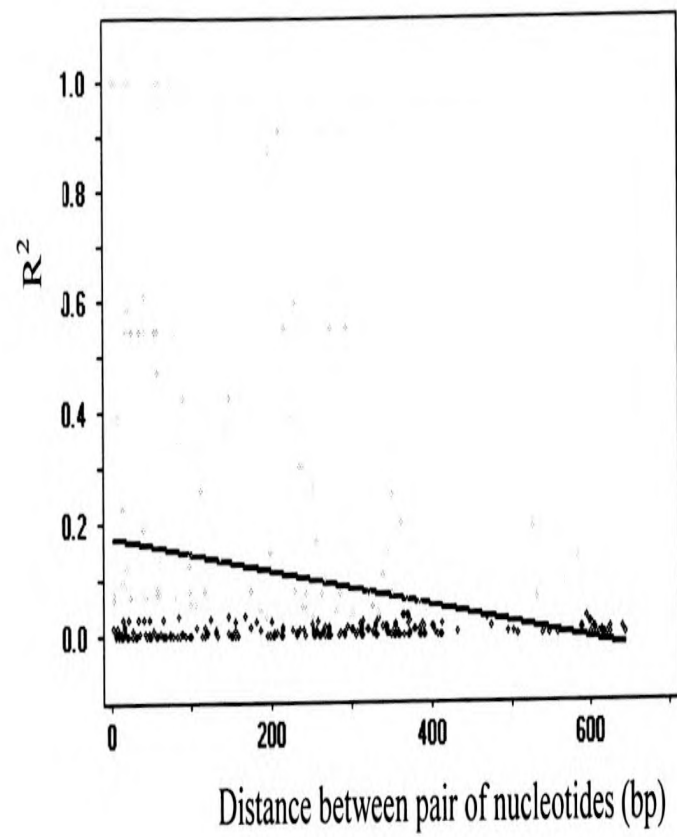
The *D'* and R^2 indices of linkage disequilibrium (LD) were also estimated across all polymorphic sites, and the relationship between LD and distance between nucleotide sites of each pairwise comparison was plotted. The statistical significance of each pairwise test of LD on these haploid data was evaluated by χ^2 analysis. All pairwise analyses are not fully independent because of LD itself, hence the proportion of significant ($P < 0.05$) pairwise test was compared for pairs of sites separated by different molecular map distance (< 200 bp or > 200 bp). The statistical significance of each pairwise test of LD of these haploid data was tested by Fisher's exact test.

Both R^2 and *D'* indices of 100 Sri Lankan sequences were declined rapidly with increasing molecular map distance (Figure 6.2). Both indices were significantly higher among sites > 200 bp apart on the chromosome with 43% of these pairwise comparisons in LD compared to sites < 200 bp apart with a lower R^2 and *D'* and significantly fewer ($P < 0.0001$; $\chi^2 = 17.55$) sites in LD (21%) (Table 6.7). Although the *C* value was relatively low, the rapid decay of LD with increasing molecular map distance is an indicator for a presence of relatively high meiotic recombination rate of the *PvdbpII* gene.

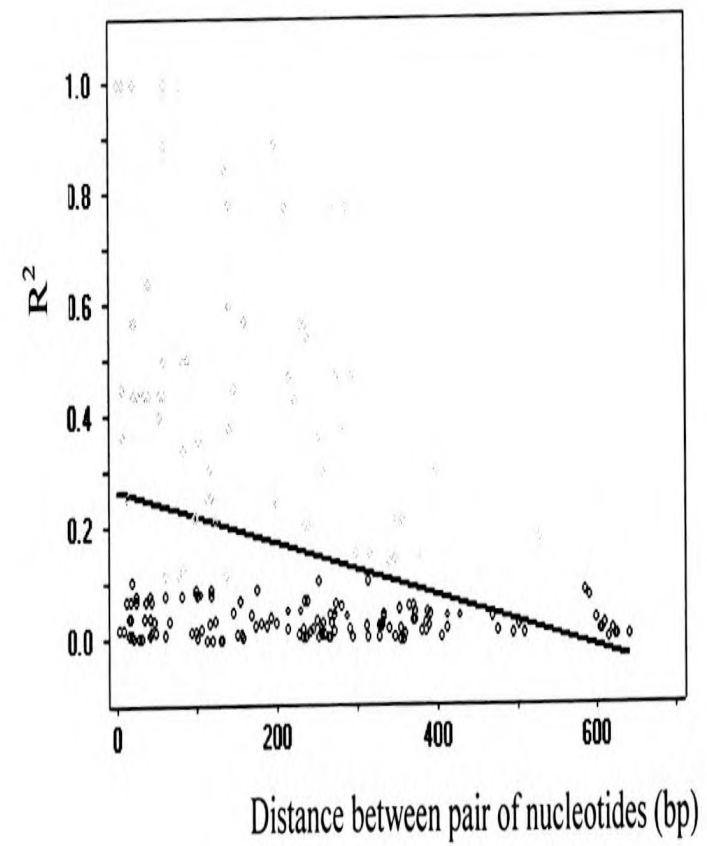
At least 7, 5 and 6 recombination events were found amongst the *PvdbpII* sequences from the three test areas Colombo, Anuradhapura and Kataragama, respectively. The *C* between the adjacent sites was estimated as 0.0262 (Colombo), 0.0246 (Anuradhapura) and 0.0073 (Kataragama), respectively. Though the R^2 values for isolates from both endemic areas declined with increasing distance, for *D'* this decline was found only for the isolates from Anuradhapura area. Indeed for isolates from Anuradhapura, the decline in LD values with molecular map distance is very steep compared to Kataragama. Both indices, R^2 and *D'*, were not significantly different among sites > 200 bp and < 200 bp apart on the chromosome for the isolates collected from Anuradhapura. However for isolates from Kataragama, a significantly higher *D'* was recorded with sites < 200 bp apart compared with sites > 200 bp apart.

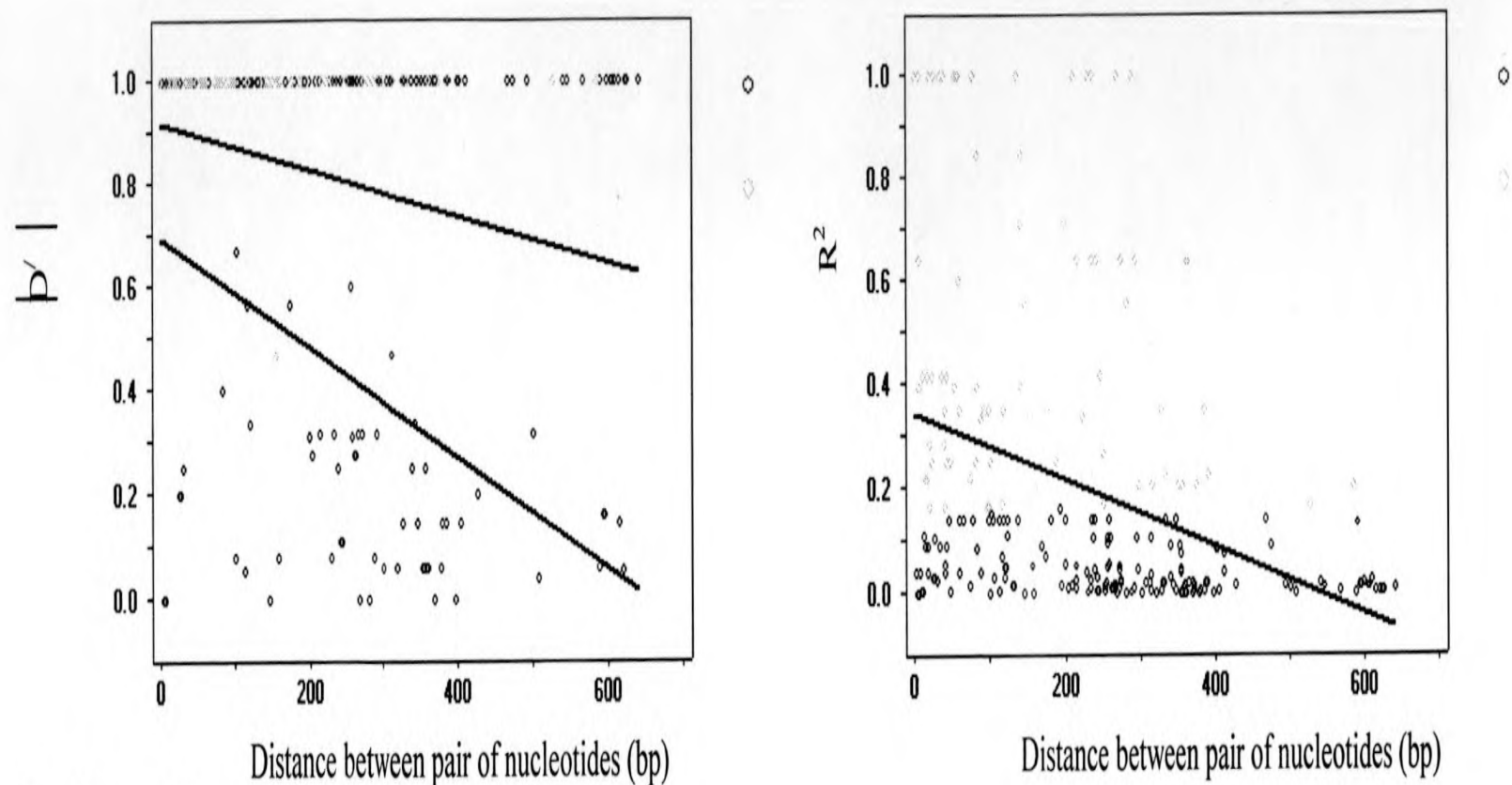


A – 100 isolates

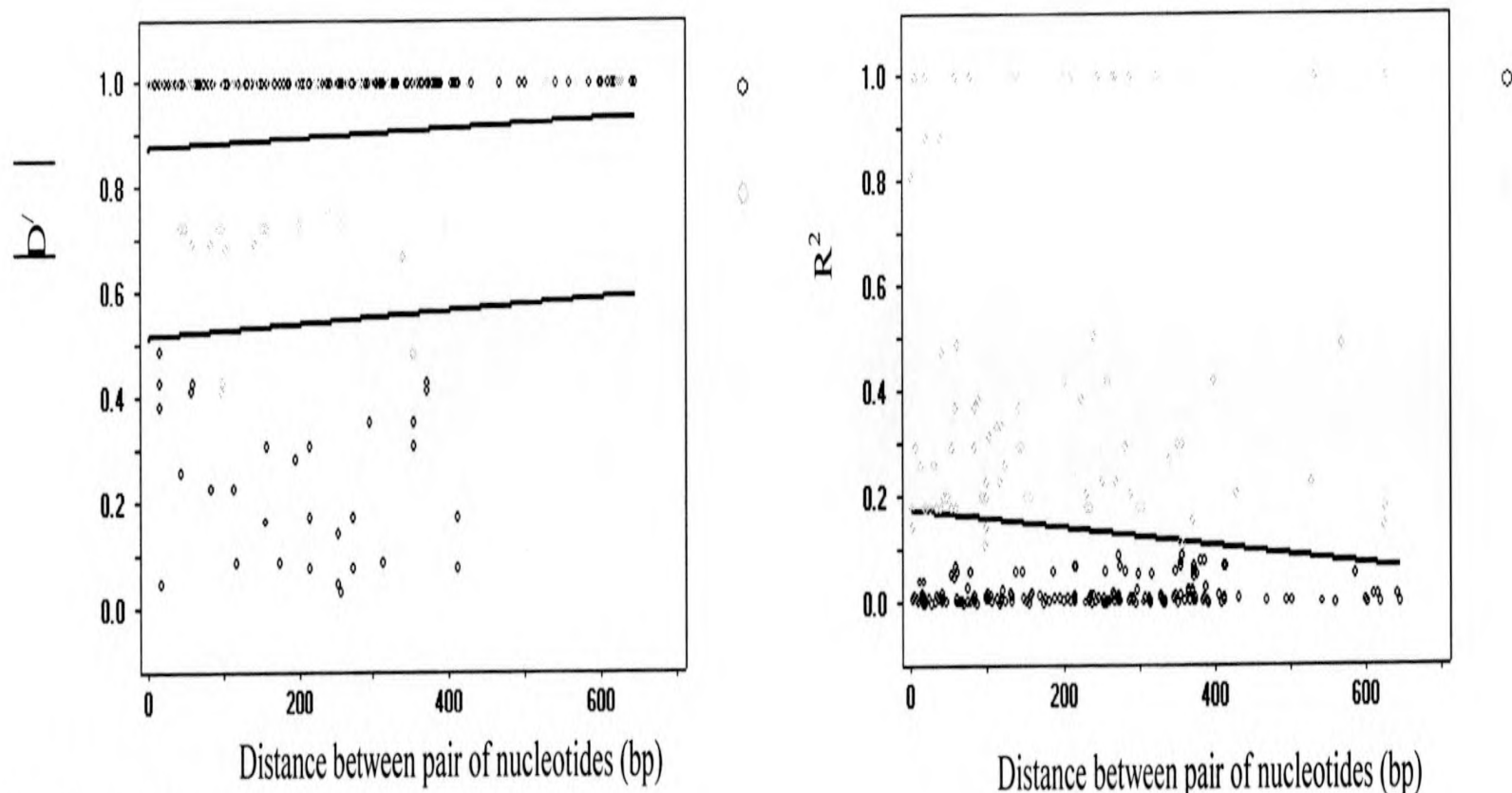


B - Colombo





C - Anuradhapura



D - Kataragama

Figure 6.2: Levels of linkage disequilibrium (LD) across *PvdbpII* in Sri Lankan field parasite isolates.

LD between all possible pairs of polymorphic sites measured using $|D'|$ (left panel) and R^2 (right panel) as a function of nucleotide distance for *PvRII* is shown. Solid circles indicate sites showing significant LD between them (Fisher's exact test) and open circles indicate non-significant LD.

A = For overall 100 isolates; B = Isolates from Colombo; C = isolates from Anuradhapura; D = Isolates from Kataragama

Table 6.7: Summary of linkage disequilibrium analysis for PvDBPII in Sri Lankan isolates

A –for all 100 isolates

	N (pairs)	$ D' $	R^2	% of significant pairwise test
< 200 bp ^a	150	0.909	0.366	43%
>200 bp ^b	198	0.861	0.268	21%
<i>P</i> vale for indices of LD ^c		0.011	0.027	

A - Isolates from Colombo

	N (pairs)	$ D' $	R^2	% of significant pairwise test
< 200 bp ^a	96	0.899	0.492	42%
>200 bp ^b	114	0.867	0.401	19%
<i>P</i> vale for indices of LD ^c		0.876	0.294	

B- Isolates from Anuradhapura

	N (pairs)	$ D' $	R^2	% of significant pairwise test
< 200 bp ^a	119	0.134	0.514	52%
>200 bp ^b	157	0.142	0.490	18%
<i>P</i> vale for indices of LD ^c		0.970	0.736	

C- Isolates from Kataragama

	N (pairs)	$ D' $	R^2	% of significant pairwise test
< 200 bp ^a	129	0.876	0.448	38%
>200 bp ^b	149	0.636	0.412	21%
<i>P</i> vale for indices of LD ^c		0.044	0.632	

N (pairs) - Number of pairwise comparisons between sites;

$|D'|$ - Average of the absolute values for D' ;

R^2 - Average of R^2 ;

% of significant pairwise tests - The percent of significance pairwise test by χ^2 analysis in each distance group.

^a Pairwise comparison between sites less than 200 bp apart

^b Pairwise comparison between sites greater than 200 bp apart

^c The probability that the mean values for indices of LD (D' and R^2) are significantly different between distance groups (<200 bp and >200 bp) as evaluated by independent sample t-test.

6.3.6. Genetic differences between parasite populations

Comparison of diversity within and between *P. vivax* populations of the *PvdbpII* sequence data from Sri Lanka in this study, and previously published sequence data from Papua New Guinea, Thailand, South Korea and Colombian populations were performed with the θ estimator of Wright's fixation index (*Fst*). The degree of genetic differentiation of *PvdbpII* gene was within the two subpopulations of the two endemic areas of Sri Lanka and amongst populations of other countries by estimating *Fst* values.

Fst values between two endemic areas of Sri Lanka, Anuradhapura and Kataragama showed a lower value - 0.103, and thus indicate a moderate genetic differentiation occurred between two sub populations. *Fst* values between overall 100 Sri Lankan isolates and most of the other countries showed high values (with Colombia *Fst* = 0.182; South Korea *Fst* = 0.190; Papua New Guinea *Fst* = 0.151). However *Fst* value between overall Sri Lankan isolates and Thailand isolates were relatively low, *Fst* = 0.095. Interestingly, the two endemic areas within Sri Lanka, Anuradhapura and Kataragama, showed a higher *Fst* value (0.103) than that between Sri Lanka and Thailand.

6.3.7. Phylogenetic analysis of the *PvdbpII* gene from geographically different malaria endemic areas

The gene tree was estimated using 672 bp region common to all of the *PvdbpII* sequences available including 100 sequences from Sri Lankan isolates and another 173 sequences previously deposited sequences from Gen Bank (111 from Papua New Guinea [PNG], 17 Colombia [Col], 13 from South Korea [SK], 30 from Thailand [Thai] and one each from India and Vietnam – Genk Bank accession numbers of these sequences are included in the cladogramme). Neighbour-joining method with Tamura three-parameter distance model used to construct the phylogenetic tree and the reliability of the N-J tree was assessed by 1000 pseudo-bootstrap replications.

Fourteen different groups in all were evident in the phylogenetic tree that included PvDBP_{II} sequences from worldwide isolates (Figure 6.3). The Sri Lankan (SL) isolates were included in 7 different clades, but were widely distributed amongst clades 7, 12 and 14 that comprised 44, 29 and 12 isolates, respectively, where evidence for clustering of the isolates according to

the sampled areas were not evident. Interestingly, of the fourteen different groups, clades 8 and 7 exclusively contained 2 and 44 isolates from SL, respectively, the only exception being a single Thai isolate in clade 7, providing evidence for close relationship of isolates from the two countries. Group 9 also contained a single SL isolate and another 2 Thai isolates only. The isolates from PNG were distributed in 8 different groups, but the majority were found in three groups 1(33%), 10 (36%) and 12 (14%). According to the cladogram, the first four clades consisted only of isolates from PNG, providing evidence for geographical isolation. However, the rest of the PNG isolates were mixed and distributed among four clades consisting of isolates from different geographical areas. Interestingly, all 13 isolates from SK tend to categorize in clade 12, which also contained 16, 8 and 29 isolates from PNG, Thai and SL, respectively, and a single isolate from Col. The single Vietnamese isolate was included in group 14 and the Indian isolate were placed in clade 10, both contained mixed isolates from different geographical regions. Sal-1 reference strain grouped in clade 10 with four SL isolates and isolates from PNG (N = 40), Colo (N = 12), Thai and India (N = 1). The SL sequences thus appeared to represent a sample from worldwide genetic diversity, rather than from any particular lineage of worldwide *PvdbpII* sequences.

6.3.8. Polymorphisms within the major linear B and T cell epitopes of PvDBPII

Using a series of overlapping peptides, major linear B-cell (Xainli *et al.* 2003) and T-cell (Xainli *et al.* 2002) epitopes associated with the 170 amino acid critical binding motif of the *PvdbpII* gene was described previously. Of these seven described epitopes, it was found that only 3 contained polymorphic residues among Sri Lankan isolates (Table 6.8). Of these, peptide 1 epitope for both B and T-cells contained 3 polymorphic residues. Further, peptide 5, a B-cell epitope contain 4 polymorphic residues, while peptide 6, a T-cell epitope contains a single polymorphic residue. Interestingly, all these polymorphic epitopes had higher nucleotide diversity value compared to that of the entire 672 bp region ($\pi = 0.0098$) analyzed (Table 6.8). Further, amino acid residues of all these polymorphic epitopes contained only non synonymous substitutions.

Identification of CD4⁺ T cell epitopes of a particular antigen which can bind and express HLA-DRB alleles is important, since polymorphism in parasite antigens, can alter the immunogenicity of that particular antigen. Saravia *et al.* (2008), assayed 10 synthetic peptides (based on Sal-1 sequence of the PvDBPII) for their ability to bind wide range of HLA-DRB alleles (10 of the most prevalent HLA-II alleles among the Asian and Afroamerican population where malaria is endemic) using peptide binding competition assays. They described two PvDBPII peptides as universal epitopes, since these two peptides were able to bind large numbers of HLA-DR molecules. According to the description, of the two universal PvDBPII epitopes, one peptide (RDYVSELPTEVQKLKEKCDG) was conserved among the 100 Sri Lankan isolates analyzed. Of the other described 20 amino acid universal epitope (ISVKNAEKVQTAGIVTPYDI), only 13 residues were sequenced in the present study, of which 39% (5 /13) were polymorphic among local isolates.

Using T-cell epitope prediction algorithm (SYFPEITHI software) (Rammensee *et al.* 1999), 15 mer peptides of the PvDBPII Sal-1 sequence were predicted and evaluated to their ability to bind by four most prevalent HLA-DRB1 alleles present in the malaria endemic population of Asia and Afroamerica. The four HAL-DRBI alleles considered was (HLA-DRB1*0101, HLA-DRB1*0401, HLA-DRB1 *0701 and HLA-DRB1*1101) (Southwood *et al.* 1998), and the binding capacity was evaluated based on a scoring system predicted by the software. Of the 224 amino acids analysed, HLA-DRB1*0101 allele was predicted to be able to bind 7 peptides of PvDBPII, HLA-DRB1*0401 6 peptides and both DRB1*0701 and DRB1*1101

alleles bind a single peptide each (Table 6.9). Some of the amino acid polymorphisms associated with some of the predicted peptides was able to change the binding capacity of that particular peptide to class II MHC allotypes. Binding of the peptide K N Q W D V L S N K F I S V K to DRB1*0101 was substantially reduced by a replacement from I (Isoleucine) to K (Lysine) (bold, italicized residue) with a score from 27 to 19. Further, polymorphisms of peptides bind to DRB1*0101, G I G Y K V V E N N L R S I (K [Lysine] to E [Glutamic acid]) reduced the score 26 to 24 and two polymorphisms of peptide N K F I S V K N A E K V Q T A (I to K and A [Alanine] to R[Arginine]) reduced the binding score 25 to 17. Polymorphism associated with peptide D T N F H R D I T F R K L Y L (F [Phenylalanine] to L [Leucine]) reduced the binding capacity of a particular peptide to DRB1*0401 from 28 to 26. Interestingly, except for the polymorphisms F to L in peptide D T N F H R D I T F R K L Y L bind to the DRB1*0401, polymorphisms (recorded in 100 Sri Lankan isolates) associated with remaining four peptides (which changes the binding capacities), resulted in amino acid class changes. However, polymorphisms associated with the remaining predicted peptides binding to four HLA-DRB1 alleles (Table 6.9), did not show reduction of the binding score. Further according to the analysis, two peptides predicted to be bind to the DRB1*0101 allele and single peptide bind to DRB1*0401 (Table 6.9) contained polymorphisms associated with the anchor residues of that particular peptide, apart from the polymorphism F to L of the peptide D T N F H R D I T F R K L Y (bind to DRB1*0401) which reduced the binding capacity from 28 to 26, other polymorphisms of the anchor residues did not change the binding capacities of those predicted peptides.

Table 6.8: Polymorphisms observed in the linear B and T-cell epitopes of the *PvdbpII* gene in Sri Lankan isolates

Peptide	Residues [#]	Epitope ^a	Sequence ^b	π
1	299-313	T / B	V <u>N</u> (Y) N T D T N <u>F</u> (L) H <u>R</u> (S) D I T F R	0.0271
2	315-329	T	L Y L K R K L Y D A A V E G	0
3	321-335	T / B	L I Y D A A V E G D L L <u>L</u> K L	0
4	329-343	T / B	G D L L <u>L</u> K L N N Y R Y N K D	0
5	380-391	B	I F G T <u>D</u> (G) <u>E</u> (K) <u>K</u> (N) A Q Q <u>R</u> (H) K Q	0.0299
6	421-435	T	I C K <u>L</u> (I) N V A V N I E P Q I Y	0.0113
7	445-459	B	Y V S E L P T E V Q K L K E K	0

[#] Residues were numbered according to Fang *et al.* 1991

^a T = T-cell epitope (Xainli *et al.* 2002); B= B-cell epitope (Xainli *et al.* 2003)

^b Bold underlined residues correspond to polymorphisms and those in the parentheses are the alternative amino acid found in the Sri Lankan field isolates as compared to the sequence of the Sal-1 strain

Table 6.9: SYFPEITHI (www.syfpeithi.de) predicted peptides of PvDBPII that bind with four HLA-DRB1 alleles

HLA-DRB1 allele type ^a	Sequence ^b	Binding Score ^c	Polymorphism in anchor residues
DRB1*0101	246 K N Q W D V L S N K F <u><i>IS</i></u> V K 260	27	No
	107 G D F G D I I M G T D M E G I 121	26	No
	120 G I G Y S K V V E N N L R S I 134	26	Yes
	196 G R D Y V S E L P T E V Q K L 210	26	No
	128 E N N L R S I F G T <u><i>DE</i></u> K A Q 142	25	No
	162 Y S V K K R L K G <u><i>NF</i></u> I W I C 176	25	No
	244 N K F <u><i>IS</i></u> V K N <u><i>AE</i></u> K V Q <u><i>TA</i></u> 268	25	Yes
DRB1*0401	57 D T N <u><i>FH</i></u> R D I T F R K L Y L 71	28	Yes
	95 N K D F C K D I R W S L G D F 109	28	No
	246 K N Q W D V L S N K F <u><i>IS</i></u> V K 260	28	No
	253 S N K F <u><i>IS</i></u> V K N <u><i>AE</i></u> K V Q <u><i>T</i></u> 267	28	No
	197 R D Y V S E L P T E V Q K L K 211	26	No
	200 V S E L P T E V Q K L K E K C 214	26	No
DRB1*0701	74 K L I Y D A A V E G D L L L K 88	30	No
DRB1*1101	158 <u><i>T</i></u> A M Y S V K K R L K G <u><i>NF</i></u> I 173	26	No

^a Four of the 10 most prevalent HLA-DRB1 alleles present in malaria endemic populations of Asia and Afroamerica (Southwood *et al.* 1998).

^b The amino acids corresponding to positions 1, 4, 6 and 9 (and 7 in some cases) were marked by SYFPEITHI in bold since they corresponded to residues fitting into the main HLA-DRB1 allele's pockets on the peptide-binding region – Anchor residues (Saravia *et al.* 2008). Underlined and Italicized residues are polymorphic sites found in the Sri Lankan isolates.

^c SYFPEITHI predicted binding score (only scores equal or more than 25 were considered)

6.3.9. Binding Inhibition Assay

A total of 162 serum samples (N = 68 from Colombo; N = 45 from Anuradhapura and N = 49 from Kataragama) were tested for the ability to inhibit the specific binding of recombinant PvRII with its corresponding red blood cell surface receptor DA, using an ELISA based binding inhibition assay. Five different dilutions (1:5, 1:10, 1:20, 1:50 and 1:100), of each serum sample were tested. For each dilution, binding inhibition was calculated and expressed as a percentage, and further the 50% binding inhibition titer was also calculated for each sample.

ELISA based binding inhibition assay revealed that serum samples from all test areas contained anti-PvDBPII binding inhibitory antibodies that exhibited inhibition at serum dilutions ranging from 1:5 to 1:100, in a dose dependent manner (Table 6.10, Figure 6.4). At the lowest serum dilution tested (1:5), 46% (75/162) of serum samples of acute *P. vivax* patients from the three test areas contained binding inhibitory antibodies. However, at increased serum dilutions, the prevalence gradually decreased, as only 27%, 16%, 5% and 2% of samples contained binding inhibitory antibodies at serum dilutions of 1:10, 1:20, 1:50 and 1:100, respectively, from the three test areas. The level of reduction of the prevalence of binding inhibitory antibodies with increasing serum dilutions was statistically significant (Chi squared test, $P < 0.001$) within each serial dilution, except at the dilutions between 1:50 and 1:100.

In the three test areas, 44%, 44% and 51% of serum samples from Colombo, Anuradhapura and Kataragama, respectively, contained binding inhibitory antibodies at a serum dilution of 1:5 (Figure 6.4). No significant difference (Chi squared test, $P > 0.05$) was apparent for the prevalence of binding inhibitory antibodies among the serum samples from malaria endemic and non-endemic areas. The same pattern was reiterated in all test areas for other serum dilutions tested.

Serum samples demonstrated different levels of binding inhibitory activities and thus were categorized in to three different groups, based on the level of percentage binding inhibition. *i.e.* samples with $>90\%$ binding inhibition activity were grouped under 'high' level, 50-89% binding inhibition as 'intermediate' level and remaining samples with $<50\%$ binding inhibition as 'low' level. Importantly it was found that 5% (N= 8) of serum samples from all three test areas contained high level of anti-PvRII binding inhibitory antibodies at serum

dilution of 1:5. Of these, three samples were found each from Colombo and Anuradhapura while the remaining 2 were from Kataragama, indicating that serum samples with high level of binding inhibitory activities were distributed both in malaria endemic as well as non endemic areas of the country. Further, 4% (2 each from the three test areas) of total serum samples showed high level of binding inhibition even at 1:10 serum dilution, though this high level of inhibition activity was not detected beyond this specific serum dilution. Of the total 162 samples, 24.6% contained intermediate level (50-89%), while the remaining 68.5% samples showed low level (<50%) of binding inhibition at 1:5 dilution. Analysis according to the three test areas revealed that 28%, 18% and 28% demonstrated intermediate level, while 68%, 76% and 67% demonstrated low level of binding inhibition in the three test areas Colombo, Anuradhapura and Kataragama, respectively. Although the prevalence of binding inhibitory antibodies from in each of the three categories in the Anuradhapura test area differed from the other two areas, the differences were not significant, and thus the prevalence of % binding inhibition at low and intermediate levels were also distributed without statistically significant differences (Chi-squared test, $P>0.05$) among the three test populations.

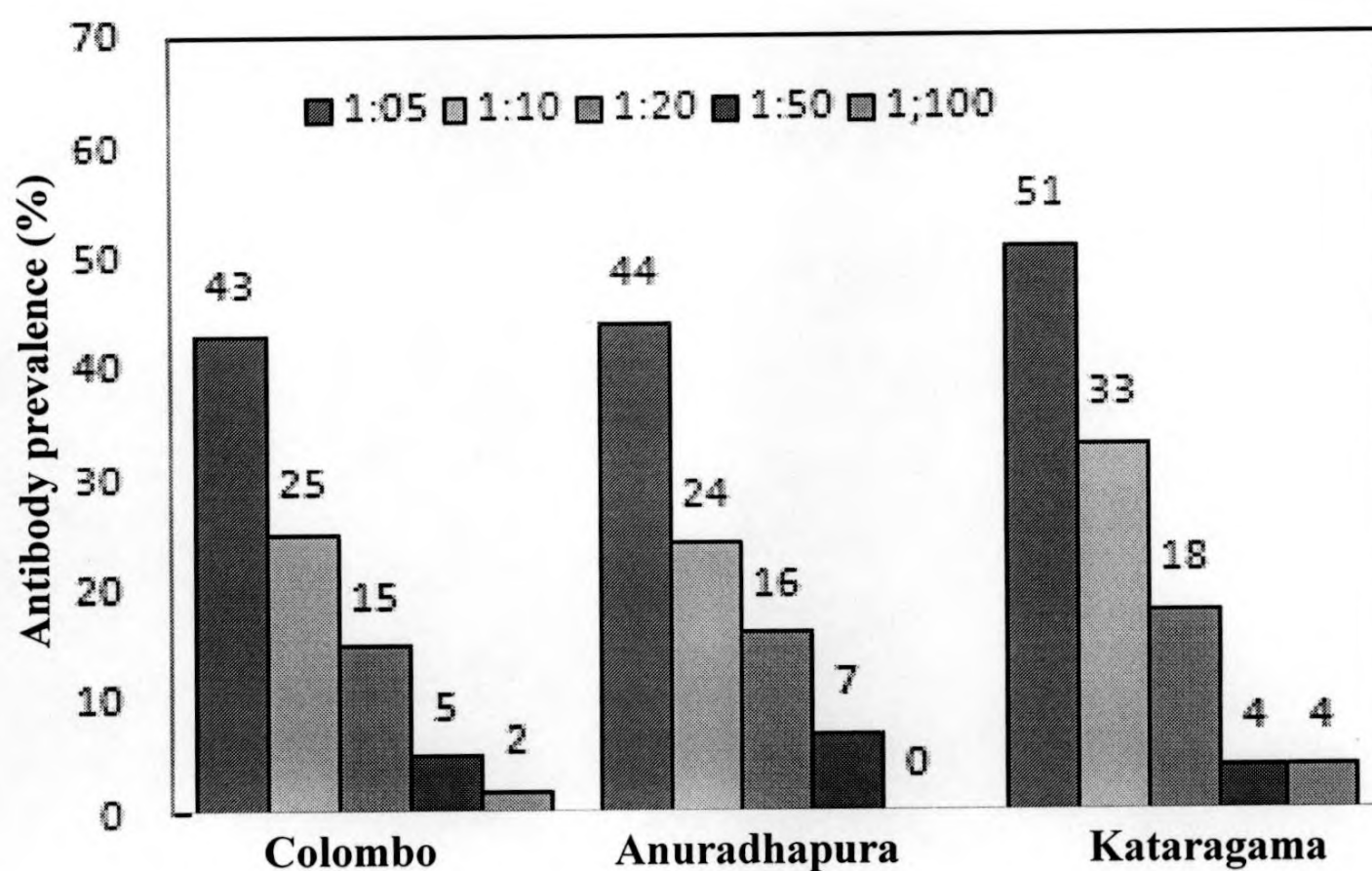


Figure 6.4: Prevalence of the anti-PvRII binding inhibition antibodies at different serum dilutions, of serum of patients of the three test areas.

Different serum dilutions tested for each serum sample are indicated in the legend of the graph. The exact values of the prevalence of anti-PvRII binding inhibitory antibodies are indicated at the top of each column.

Serum samples N = 68 from Colombo; N = 45 from Anuradhapura and N = 49 from Kataragama, were used for the assay.

Table 6.10: Prevalence and percentage inhibitions of anti-PvRII binding inhibitory antibodies at different serum dilutions of sera from acute *P. vivax* malaria patients from the three study areas

Serum dilution	1:05		1:10		1:20		1:50		1:100	
	Prevalence (%)	% Inhibition (SEM)	Prevalence (%)	% Inhibition (SEM)	Prevalence (%)	% Inhibition (SEM)	Prevalence (%)	% Inhibition (SEM)	Prevalence (%)	% Inhibition (SEM)
Total (N=162)	46	62.0 (2.25)	27	53.57 (2.97)	16	43.86 (3.26)	5	33.79 (3.83)	2	31.01 (2.43)
Colombo (N=68)	43	60.0 (3.32)	25	53.82 (4.22)	16	47.75 (5.41)	5	38.26 (10.03)	2	32.99
Anuradhapura (N=45)	44	64.83 (5.05)	24	60.23 (6.48)	16	48.63 (8.28)	7	28.18 (2.29)	-	-
Kataragama (N=49)	51	62.57 (3.79)	33	48.72 (5.13)	18	35.81 (2.64)	4	35.50 (3.64)	4	30.03 (3.86)

For the total 48 serum samples that showed high and intermediate levels of binding inhibition at 1:5 serum dilution, the exact 50% binding inhibition titer was calculated. The mean value of the 50% binding inhibition titer for overall serum samples was 12.55 (± 1.43), while for the three test areas separately the values were 11.44(± 2.3) for Colombo, 16.35(± 3.2) for Anuradhapura and 11.46(± 2.06) for Kataragama. Although not significant (Kruskal-Wallis test, $P > 0.05$) the mean value of 50% binding inhibition titer was high in endemic area Anuradhapura compared to Colombo and Kataragama.

Magnitudes of total (IgG+IgM) and IgG specific anti-PvRII antibodies significantly correlated with corresponding binding inhibition titers at 1:5 serum dilution and 50% binding inhibition titers of the serum samples from two endemic areas (Spearman's correlation coefficient; $P < 0.05$) (Table 6.11). In non endemic Colombo, this correlation was significant only between total and IgG antibodies with 50% binding inhibition titer.

Anti-PvRII binding inhibition antibody titers did not exhibit significant associations with age and days of symptoms of the malaria patients from the three test areas. PE patients from non-endemic Colombo exhibited significantly higher prevalence (Chi square test, $P > 0.05$) of anti-PvRII binding inhibitory antibodies at serum dilutions 1:5, 1:10 and 1:20. Although not significant (Mann-Whitney U test, $P > 0.05$) 50% inhibition titer of the PE group were higher than that of PNE group. Interestingly none of the PNE patients from two endemic areas contained binding inhibitory antibodies at any of the serum dilutions we tested. Further, though significant (Chi square for linear trend, $r = 7.35$; $P = 0.006$) only in non endemic Colombo, with increasing exposure, a parallel increase of the prevalence of anti-PvRII binding inhibitory antibodies (at 1:5 serum dilution) were evident in all there test areas (Figure 6.5). Further, in two endemic areas, though not significant, a negative correlation was observed for anti-PvRII binding inhibitory antibody titers with % parasitaemia (parasite load) of the serum of acute *P. vivax* patients, at the time of blood collected (Figure 6.6).

Table 6.11: Correlation between anti-PvRII binding inhibition titers with anti-PvRII Total (IgG+IgM) and IgG antibodies in serum from acute *P. vivax* patients of the three test areas

Area		Titer at 1:5 serum dilution	50% binding inhibition titer
Colombo	Total	29 ¹	29
		0.132 ² (0.286) ³	0.362 (0.050)
	IgG	25	25
		0.381 (0.060)	0.431 (0.031)
Anuradhapura	Total	20	20
		0.541 (0.014)	0.579 (0.007)
	IgG	17	17
		0.603 (0.010)	0.564 (0.017)
Kataragama	Total	24	24
		0.612 (0.001)	0.543 (0.006)
	IgG	23	23
		0.694 (0.001)	0.719 (0.001)

¹Number of samples

²Spearman's correlation coefficient

³P value

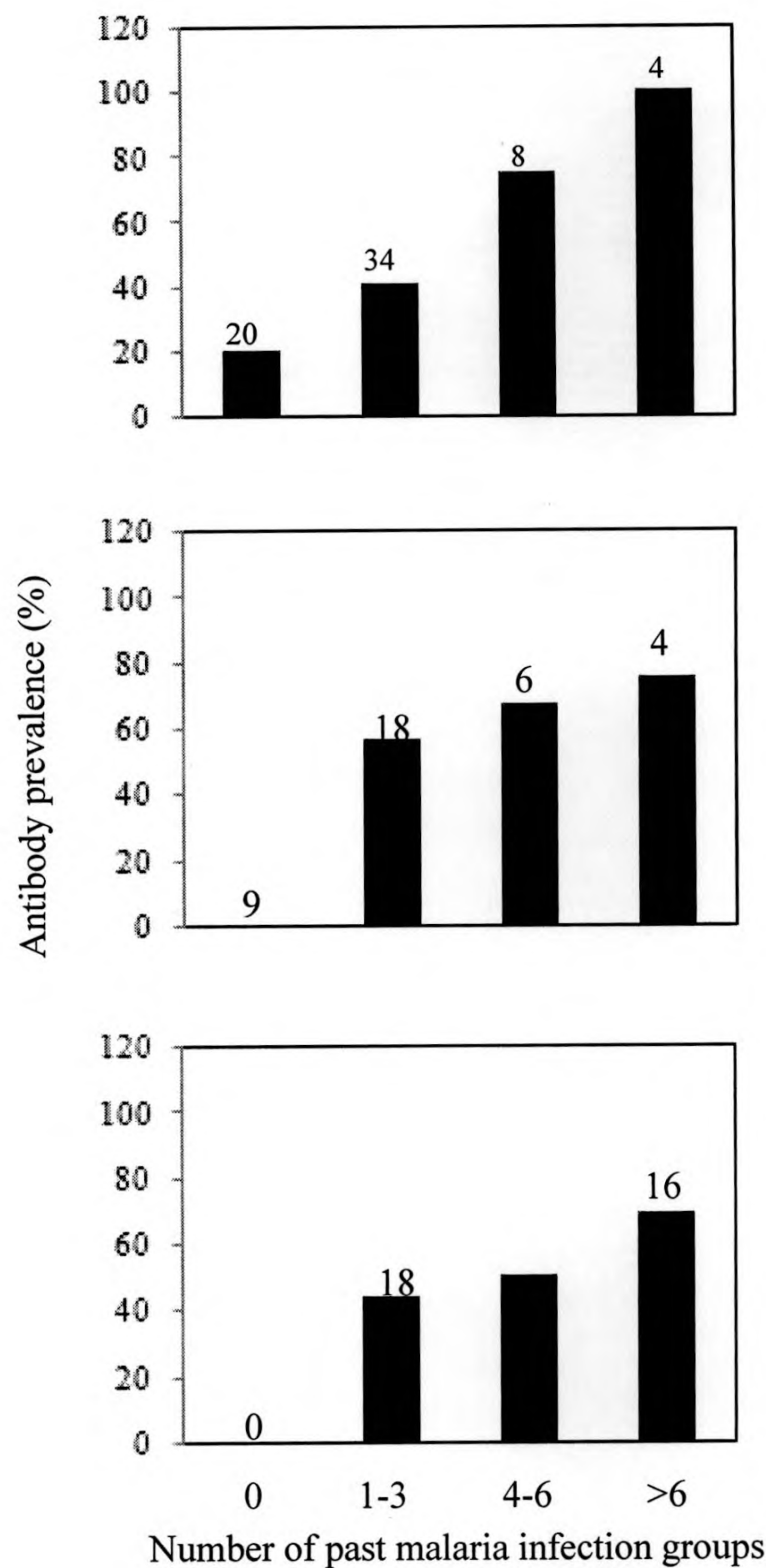
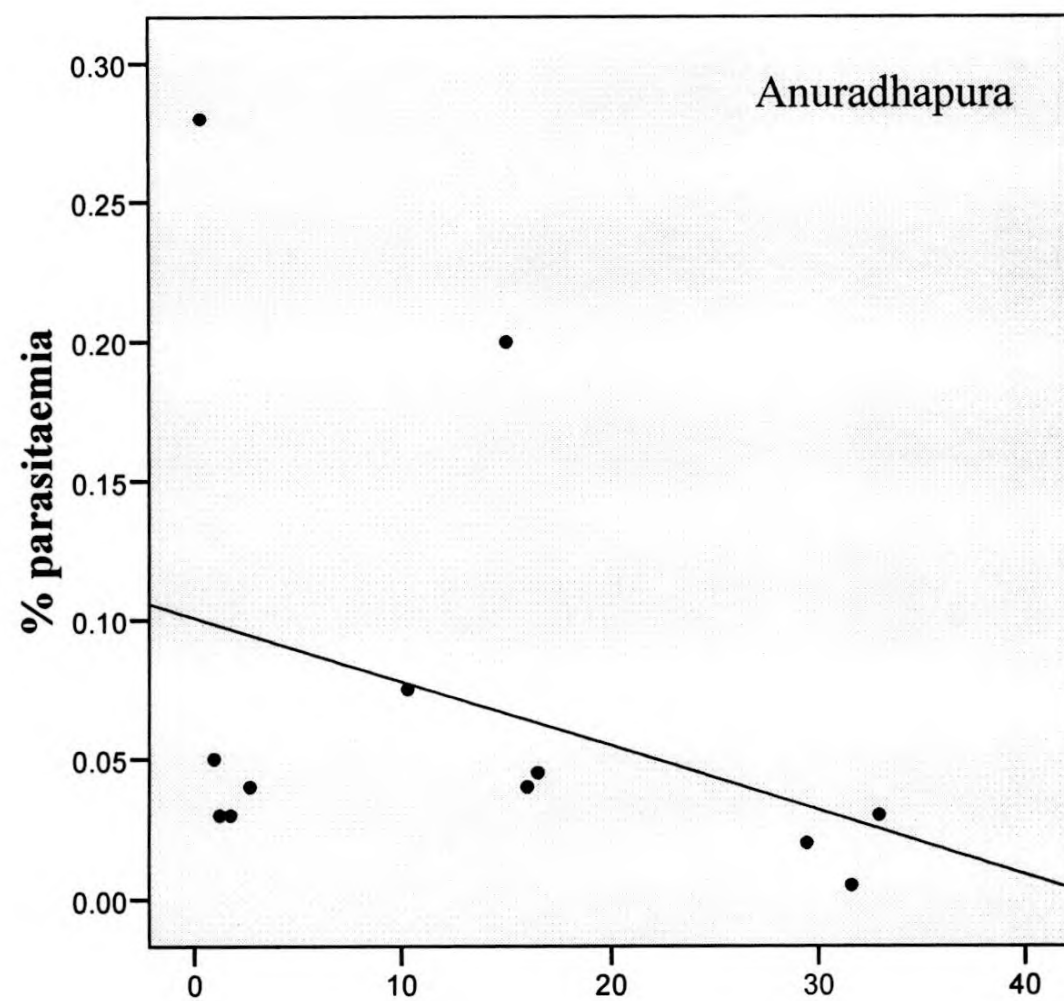


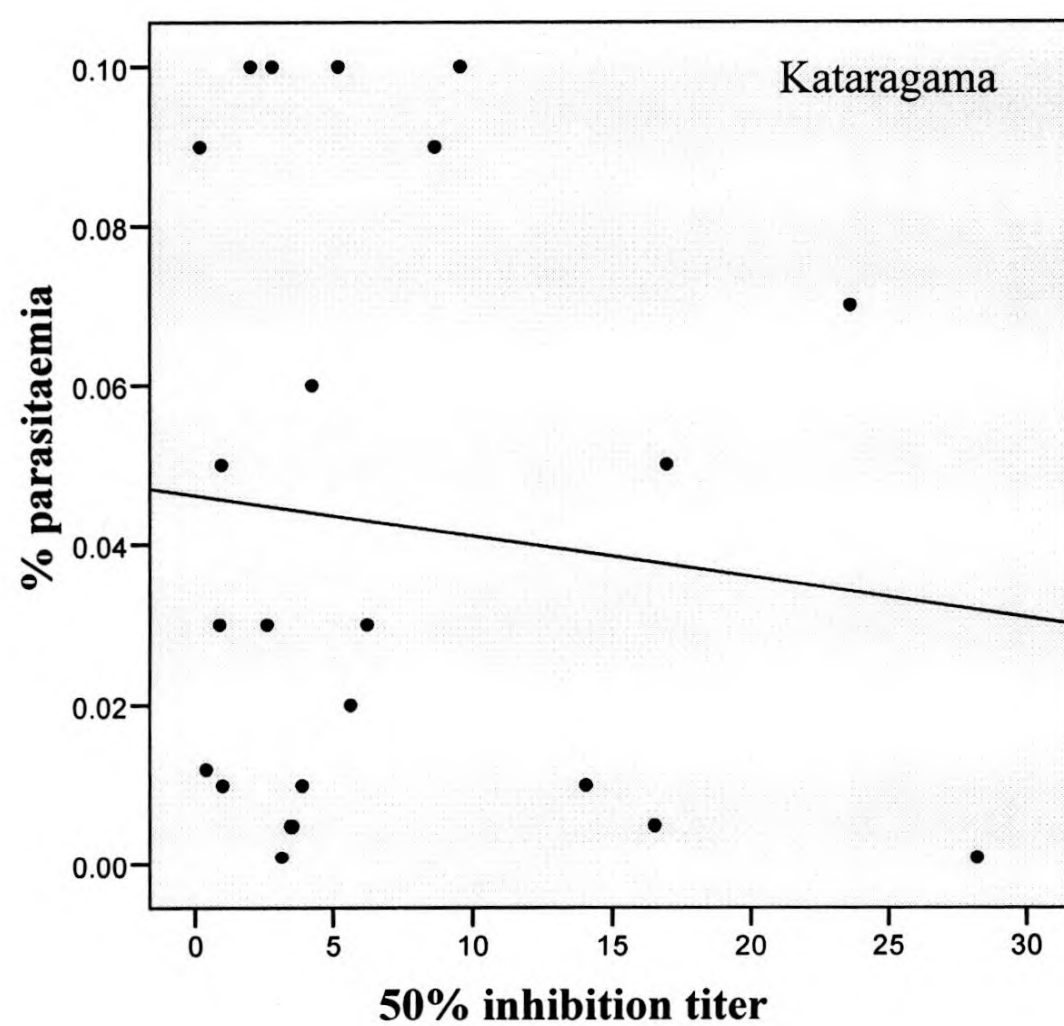
Figure 6.5: Prevalence of anti-PvRII binding inhibitory antibodies (at 1:5 serum dilution) in patients of different malaria exposure groups from the three test areas.

The number of individuals tested in each past exposure group is indicated above the bars.

0 = Patients with no past malaria infections (PNE); 1-3 = Patients with 1-3 past malaria infections; 4-6 = Patients with 4-6 past malaria infections; >6 = Patients with more than 6 past malaria infections



Speaman's correlation coefficient
 $r = -0.347$; $P = 0.269$



Speaman's correlation coefficient
 $r = -0.092$; $P = 0.675$

Figure 6.6: Correlation of 50% binding inhibitory antibody titers of anti-PvRII IgG specific antibodies with the % parasitaemia at the time of blood collection of the acute *P. vivax* patients from the two endemic areas

6.3.10. Strain-specific and strain-transcending antibody responses directed against PvDBP_{II}

In order to identify any existing correlation between naturally acquired anti-PvDBP_{II} antibody responses with different PvDBP_{II} amino acid haplotypes prevalent in the field parasite isolates within Sri Lanka (Table 6.12), different PvDBP_{II} amino acid haplotypes were aligned with the homologous total (IgM+IgG) and IgG isotype specific antibody responses as well as binding inhibitory antibody responses. *(The relevant antibody mediated immune response data was obtained from parallel research conducted with the supported by the National Science Foundation Scholarship grant – NSF/Sch/2004/07).*

It was evident that naturally acquired antibodies against different PvDBP_{II} strain types, recognized recombinant protein PvR_{II} that was based on PvDBP_{II} Sal-1 strain. Of the 100 PvDBP_{II} sequences analyzed, only one (SL 18) contained identical amino acid sequence to the PvDBP_{II} Sal-1 strain, while other strains showed amino acid homology to the Sal-1 strain ranging from 95.536% - 99.554% (Table 6.12). Although the % homology is identical for some of the different strains on the basis of the number of different amino acids compared to the Sal-1 strain (eg: SL-3 and SL-6 [98.241%]; SL-5, SL-11, SL-25, SL-26, SL-29 and SL-31 [95.58%], each of these strains contained its own specific amino acid sequences (Table 3.4.12). Of the 21 serum samples corresponding to the PvDBP_{II} SL-1 type parasite strain, with a 98.7% homology to the Sal -1 strain, 62% contained detectable total (IgM+IgG) antibodies against recombinant protein based on PvDBP_{II} Sal-1 strain. Likewise, 69%, 87%, 83% and 100% of serum samples corresponding to the SL-2, SL-3, SL-4 and SL-5 strain types, respectively, also contained detectable levels of total antibodies against the Sal-1 strain (Table 6.12, Figure 6.7). This clearly indicates that naturally acquired anti-PvDBP_{II} antibodies are strain transcending, at least cross reacting against two different strains. In other words this indicates that serum samples of some individuals contained anti-PvDBP_{II} cross reactive antibodies while some individuals did not. No detectable association was found between % amino acid homology (to the Sal-1) of the different PvDBP_{II} stains with the corresponding magnitudes of the naturally acquired total antibodies. Similar results were obtained for naturally acquired anti-PvDBP_{II} IgG specific antibodies with 92%, 82%, 69%, 80% and 40% serum samples corresponding to SL-1, SL-2, SL-3, SL-4 and SL-5 strains, respectively, against the PvDBP_{II} Sal-1 strain (Table 6.12; Figure 6.8).

However, magnitudes of the anti-PvRII total (IgM+IgG) antibodies varied according to the relative frequencies of the corresponding strain types. Highest magnitude of total (IgM+IgG) antibodies (mean absorbance = 0.778) was recorded for the most prevalent strain type, the SL-1 followed by SL-2, 3, 4, 5 and 6 (Figure 6.9). Thus apart from strain transcending antibody response, this clearly indicated the existence of a strain specific antibody response to PvRII in Sri Lanka. However, evidence for strain specific antibody response was not recorded for anti-PvRII IgG antibodies, since the IgG responses did not vary according to the frequency of the corresponding strain type (Figure 6.10).

Table 6.12: Naturally acquired anti-PvRII total (IgM+IgG) and IgG isotype specific antibody responses of the different PvDBPII amino acid Sri Lankan *P. vivax* amino acid haplotypes analysed against the PvDBPII Sal-1 strain

*PvDBPII Strain types (described in Table 6.2)

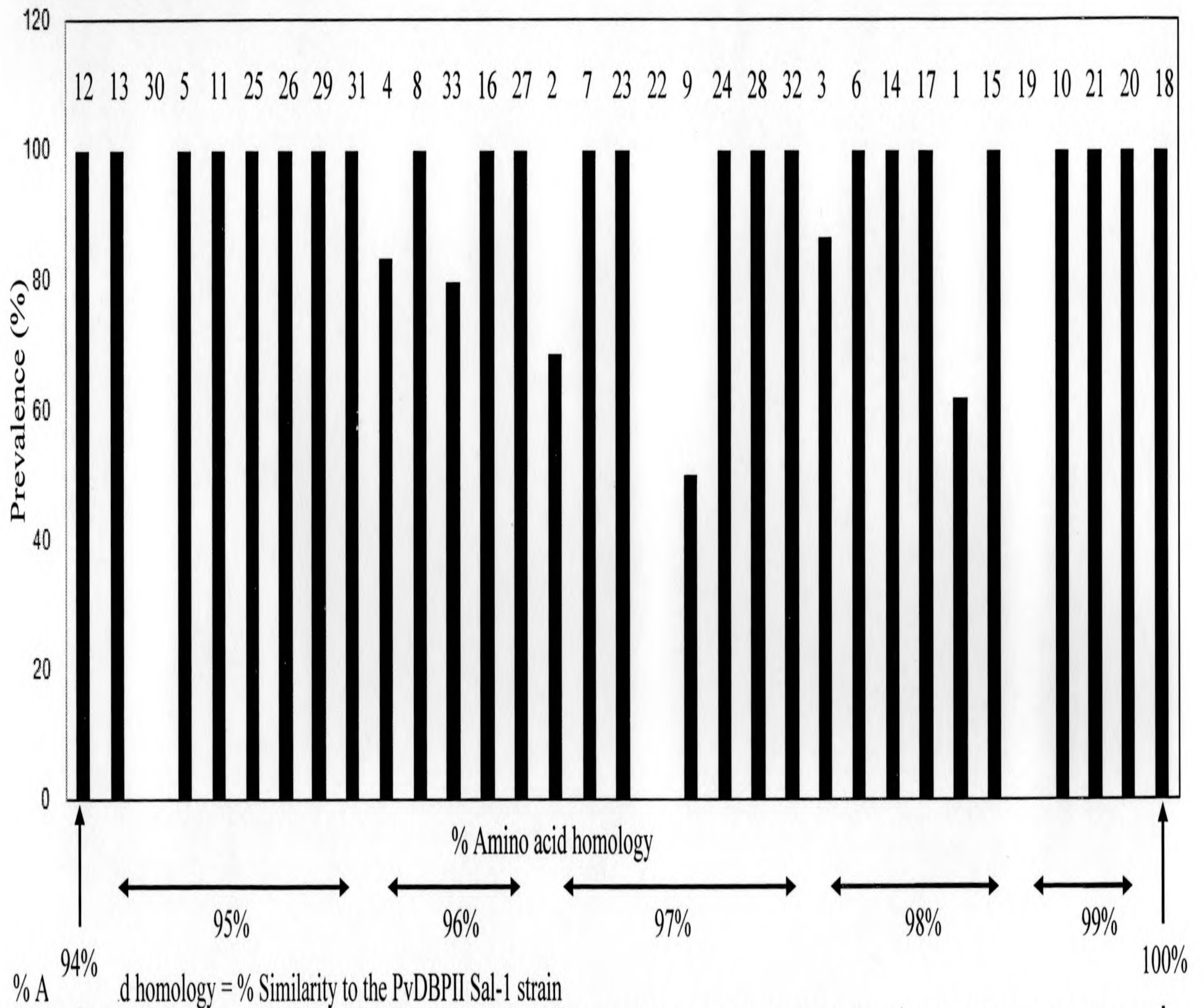
PvDBPII Strain*	% Homology [#]	Anti-PvRII total (IgM+IgG)		Anti-PvRII IgG	
		Prevalence (N)	Magnitude (±S.E)	Prevalence (N)	Magnitude (±S.E)
SL 1	98.661	62 (21)	0.778 (±0.12)	92 (13)	0.388 (±0.04)
SL 2	97.321	69 (16)	0.748 (±0.11)	82 (11)	0.481 (±0.07)
SL 3	98.214	87 (15)	0.793 (± 0.20)	69 (13)	0.407 (±0.02)
SL 4	96.429	83 (6)	0.780 (±0.12)	80 (5)	0.447 (±0.04)
SL 5	95.982	100 (5)	0.520 (±0.06)	40 (5)	0.364 (±0.02)
SL 6	98.214	100 (4)	0.505 (±0.05)	25 (4)	0.267
SL 7	97.321	100 (3)	0.960 (±0.46)	100 (3)	0.481 (±0.08)
SL 8	96.429	100 (3)	0.619 (±0.22)	67 (3)	0.508 (±0.27)
SL 9	97.768	50 (2)	0.368	100 (1)	0.488
SL 10	99.107	100 (2)	0.418 (±0.05)	100(3)	0.312 (±0.02)
SL 11	95.982	100 (1)	0.582	0 (1)	
SL 12	94.643	100 (1)	0.501	100 (1)	0.515
SL 13	95.536	100 (1)	0.964	100 (1)	0.969
SL 14	98.214	100 (1)	0.364	100 (1)	0.319
SL 15	98.661	100 (1)	0.348	100 (1)	0.288
SL 16	96.875	100 (1)	0.584	0 (1)	
SL 17	98.214	100 (1)	0.622	100 (1)	0.290
SL 18	100.0	100 (1)	1.27	100 (1)	0.335
SL 19	99.107	100 (1)	0.823	100 (1)	0.616
SL 20	99.554	0 (1)			
SL 21	99.107	100 (1)	0.514	0 (1)	
SL 22	97.768	100 (1)	0.643	100 (1)	0.500
SL 23	97.321	0 (1)			
SL 24	97.768	100 (1)	1.18	100 (1)	0.514
SL 25	95.982	100 (1)	0.471	0 (1)	
SL 26	95.982	100 (1)	1.94	100 (1)	0.993
SL 27	96.875	100 (1)	0.542	100 (1)	0.692
SL 28	97.768	100 (1)	0.485	100 (1)	0.358
SL 29	95.982	100 (1)	0.779	100 (1)	0.519
SL 30	95.982	100 (1)		0 (1)	
SL 31	95.982	0 (1)	1.22		
SL 32	97.768	100 (1)	0.384	100 (1)	0.302
SL 33	96.875	100 (1)	0.377	100 (1)	0.309

[#]% Homology - Homology compared to the PvDBPII Sal-1 strain

Prevalence = % Antibody prevalence (N = Number of isolates)

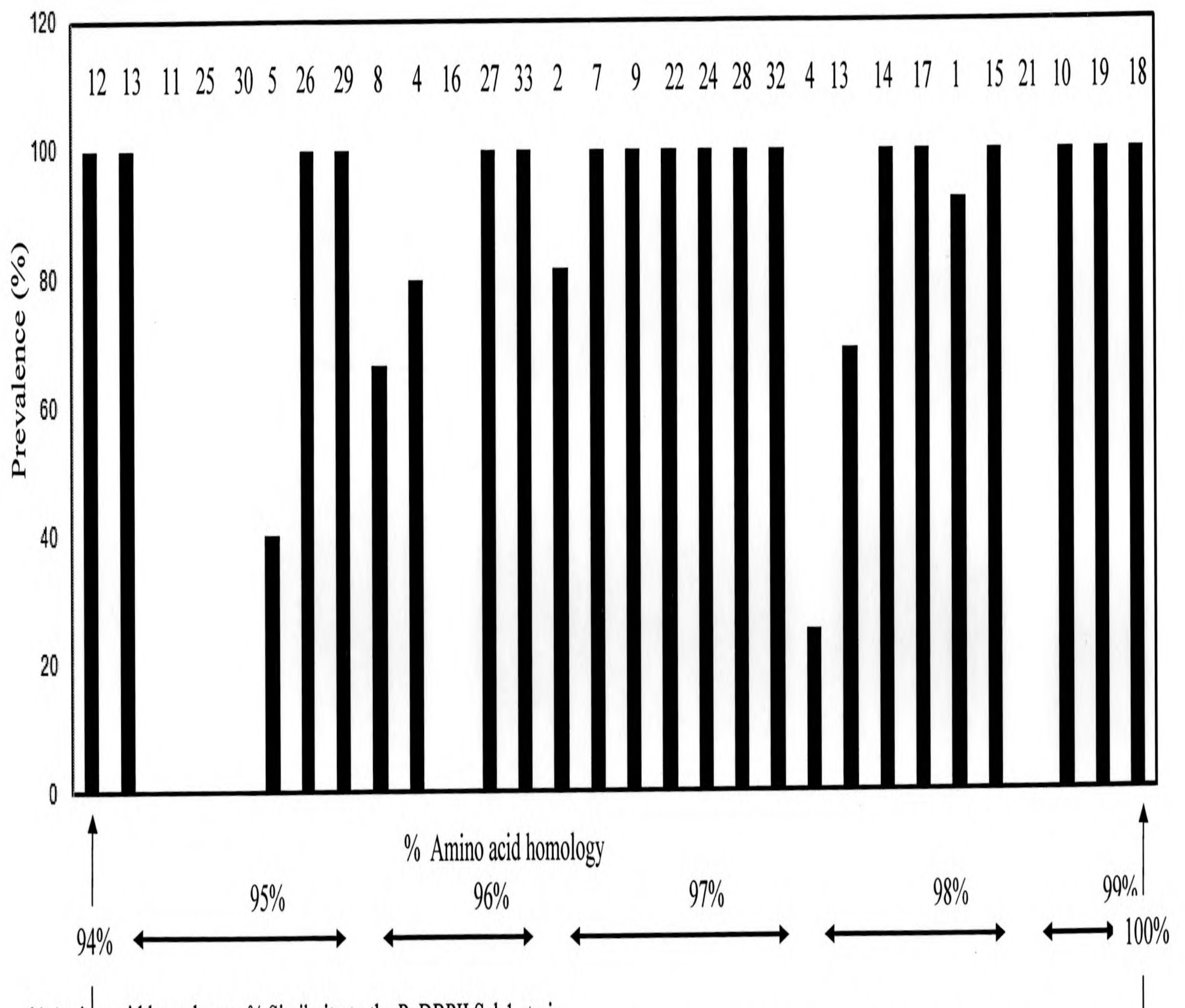
Magnitude = Average magnitude of the positive responders (S.E. = Standard error)

Figure 6.7.: Prevalence of anti-PvRII total (IgM + IgG) antibody responses with respect to the % amino acid homology of the corresponding *P. vivax* parasite isolate



Numbers above the bars corresponding to the different PvDBPII strain types (SL 1 – SL 33) recorded in the *P. vivax* field isolates in Sri Lanka (Described in Table 6.2).

Figure 6.8.: Prevalence of anti-PvRII IgG antibody responses with respect to the % amino acid homology of the corresponding *P. vivax* parasite isolate



% Amino acid homology = % Similarity to the PvDBPII Sal-1 strain

Numbers above the bars corresponding to the different PvDBPII strain types recorded in the *P. vivax* field isolates in Sri Lanka (Amino acid differences of the each of the strains were described in table 6.12).

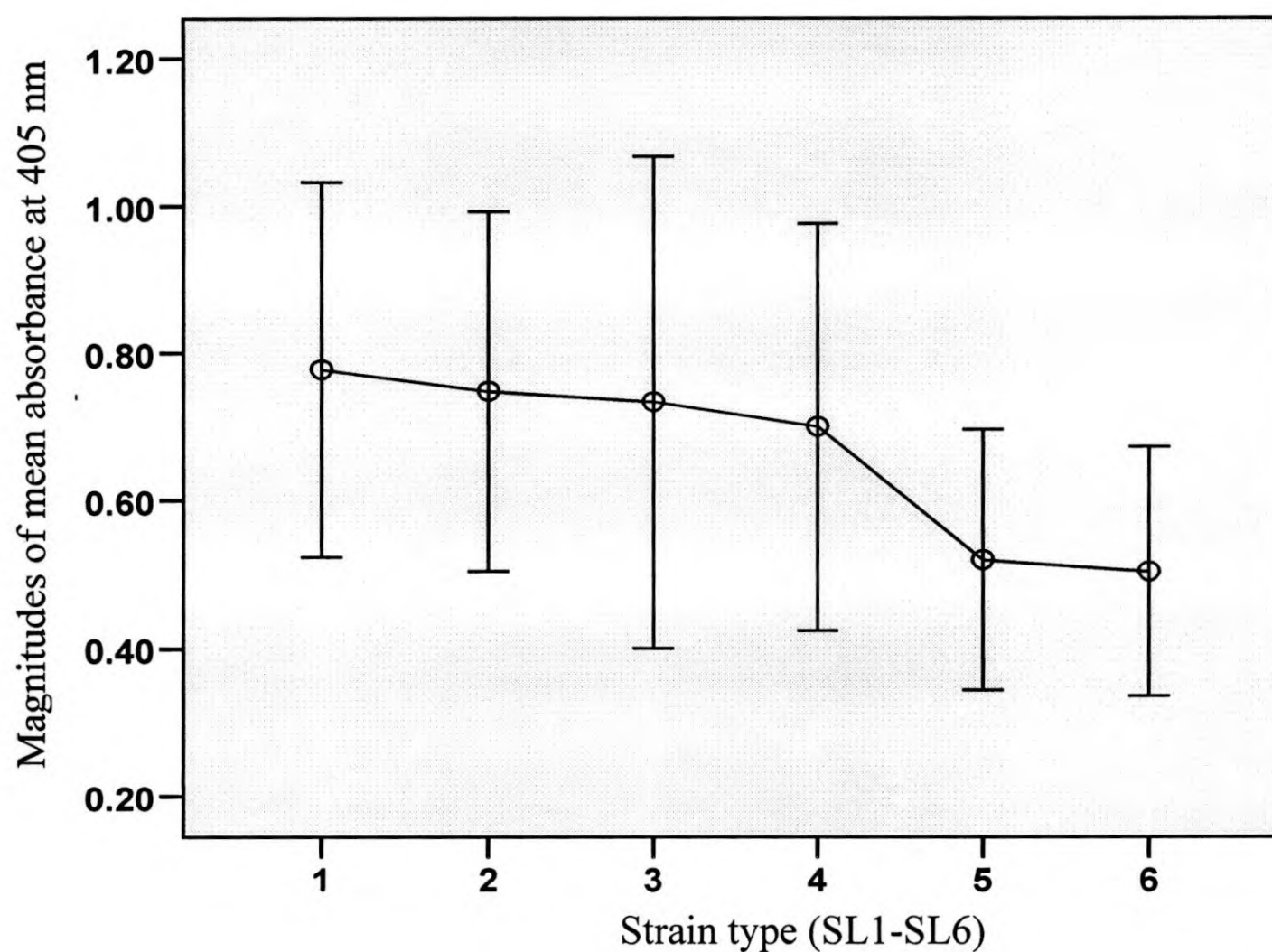


Figure 6.9.: Mean values of the magnitudes of anti-PvR11 total (IgM+IgG) antibodies according to the corresponding strain type of the six most prevalent PvDBP11 strains recorded in Sri Lanka

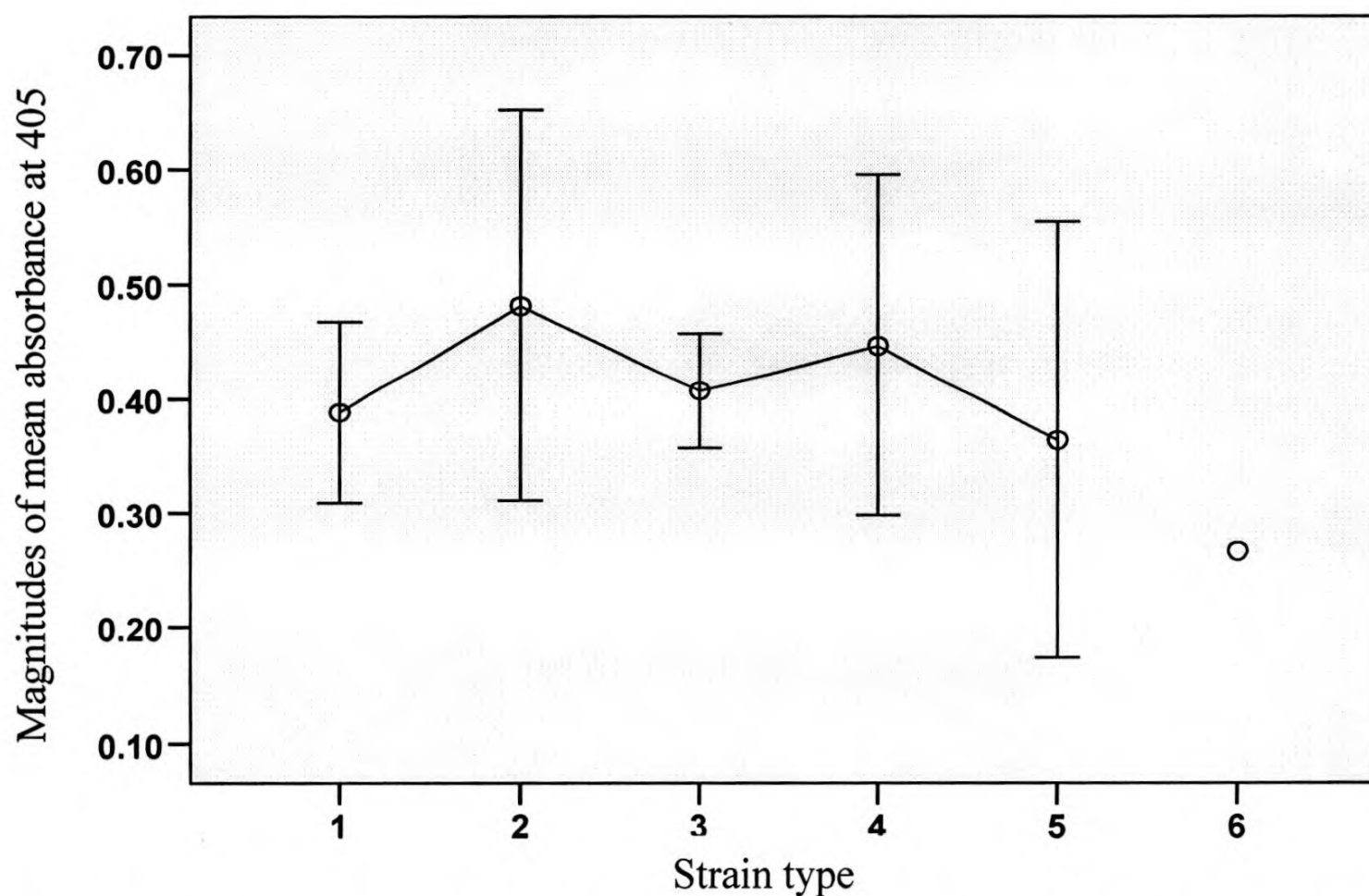


Figure 6.10.: Mean values of the magnitudes of anti-PvR11 IgG antibodies according to the corresponding strain type of the six most prevalent PvDBP11 strains recorded in Sri Lanka

Genetic complexity of *Plasmodium vivax* infections in Sri Lanka, as reflected at the merozoite-surface-protein-3 α locus

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Received 14 December 2009, Revised 29 January 2010,

Accepted 3 February 2010

The presence of genetically different strains of malarial parasites in cases of human malaria is a severe drawback in the successful control of the disease. In Sri Lanka, although this species accounts for 60%–80% of all of the cases of clinical malaria that occur each year, the genetic complexity of *Plasmodium vivax* on the island remains to be elucidated. In recent studies based on PCR–RFLP and the parasites' merozoite-surface-protein-3 α locus, the genetic structure of 201 clinical isolates of *P. vivax*, from two malaria-endemic areas and a non-endemic area of the island, was investigated. Although the PCR only produced amplicons of three sizes [1900 (72.6%), 1500 (25.9%) and 1200 (1.5%) bp], the RFLP analysis based on *Hha*I or *Alu*I digestion yielded 22 and 26 restriction patterns, respectively, with 51 combined patterns recorded. The distribution of the prominent PCR–RFLP haplotypes was area-specific. The probability that an investigated case had a multiple-clone infection (MCI) was higher among the cases from the endemic areas (20.0%) than among those from the non-endemic area (13.8%) but this difference was not statistically significant. Since 17 single-clone isolates produced only 11 different PCR–RFLP haplotypes but (after sequencing) 13 distinct nucleotide haplotypes, it is clear that the results of the PCR–RFLP were not revealing all of the diversity that existed at the nucleotide level. Four mass blood surveys in a malaria-endemic area demonstrated that seasonal changes in the prevalences of human infection with *P. vivax* may influence the occurrence of MCI.

A host may carry a genetically complex or homogeneous infection with a single species of parasite. Exploration of the genetic diversity of parasite populations is a prerequisite for understanding the relevant molecular epidemiology, developing effective vaccines and drugs and planning vaccination and therapeutic strategies (Cui *et al.*, 2003).

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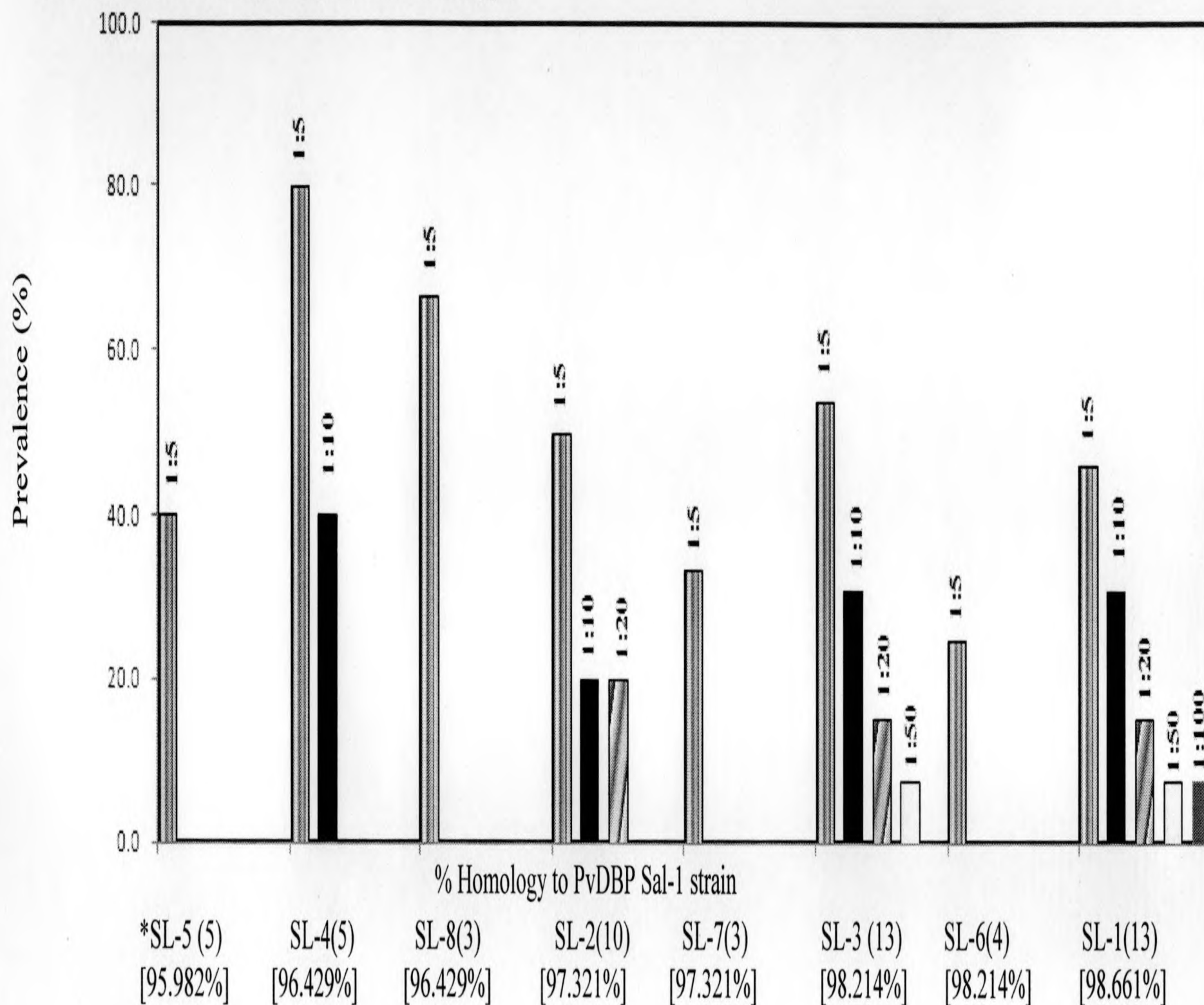
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Malarial parasites are haploid except during a brief period of sexual recombination in the mosquito vector. The infection of a single mosquito with multiple strains of *Plasmodium* results from the mosquito feeding either on a single host that carries more than one parasite genotype or, as the result of interrupted feeding, on multiple hosts within one gonotrophic cycle (Druilhe *et al.*, 1998). In humans, multiple-clone infections (MCI) with malarial parasites may result not only from a single bite of a mosquito that has a multiple-clone infection itself but also from relapse infections and super infections in the human hosts (Havryliuk and Ferreira, 2009). The degree of genetic heterogeneity seen in both *P. vivax* and *P. falciparum* infections in humans has been

Attempts were also made to establish relationship between the prevalence of cross reactive antibodies with intrinsic properties of different PvDBPII strains. The five most prevalent PvDBPII strains contained 2,6,2,8 and 9 variant amino acids, respectively, compared to the Sal-1 strain. Further each of these five strains contained 1,4,1,6 and 5 amino acid variants, respectively, which resulted in an amino acid class difference compared to the Sal-1 strain. However, no significant association was evident between the prevalence of cross reactive antibodies with each of these intrinsic properties of the PvDBPII strains.

Evidence for strain specificity of naturally acquired antibody responses were also detected by analyzing patients who experienced their first malaria infections (malaria naïve individuals) from non-endemic Colombo. Three patients each, without previous malaria infections, infected with strain types SL 1 and SL 2 (two most predominant strain types recorded in the present study), did not show evidence for the presence of cross reactive antibodies, since all six individuals were negative responders for total (IgG+gM) and IgG isotype specific antibody responses as well as anti-PvRII binding inhibitory antibodies at any given serum dilution tested. Similar type of analysis was not possible for antibody responses from the two endemic areas, due to the lack of naïve individuals in these study populations. More importantly, naturally acquired antibodies against a particular PvDBPII strain, effectively blocked the binding of recombinant PvRII (based on PvDBP Sal-1 strain) with its corresponding erythrocyte receptor, the Duffy antigen. Of the 26 PvDBPII strain types of which the corresponding binding inhibitory antibody responses were tested, 62% (16 / 26) of PvDBPII SL strains contained binding inhibitory antibodies with the ability to cross react with the PvDBPII Sal-1 strain. Prevalence of the anti-PvRII binding inhibitory antibodies at different serum dilutions showed an association with the % amino acid homology of the corresponding strain type. Isolates of the PvDBPII SL-1 strain, with a 98.661% homology to Sal-1 type (Table 6.12), contained corresponding binding inhibitory antibodies up to a serum dilution of 1:100 (Figure 6.11). However, SL-3 and SL-6, both with 98.214% homology to Sal-1 contained binding inhibitory antibodies only up to 1:5 and 1:50 dilutions respectively (Figure 6.11.). Likewise, SL-5 with only 95.982 % homology contained binding inhibitory antibodies only at the highest serum dilution of 1:5. However, SL-18 with 100% identical amino acid sequence to the Sal-1 strain did not contain detectable level of binding inhibitory antibodies. These results clearly indicate that, naturally acquired antibodies, in terms of binding inhibition, is effective despite the presence of different PvDBPII strains, which is an important issue in terms of vaccine development based on the *P. vivax* Duffy binding protein.

Figure 6.11.: Prevalence of the anti-PvRII binding inhibitory antibodies at different serum dilutions with respect to % homology to the Sal-1 strain of the corresponding PvDBPII strain types



*SL numbers are the different strain types and numbers within parentheses are the number of samples used for the calculations.

[Percentage homology of the SL strains to the PvDBP Sal-1 strain]

6.4. Discussion

The efficacy of vaccines based on polymorphic antigens is largely influenced by the local host immune responses. As such, the characterization of antigenic diversity among locally prevalent parasite strains and studying the ensuing antibody mediated strain specific immunity will be particularly important in a specific geographic setting. To date, there is no report of the comprehensive analysis of genetic diversity of the region II of the *P. vivax* Duffy binding protein (*PvdbpII*) gene, particularly in the Indian sub continent, including Sri Lanka. Thus, during the study we attempted to characterize the prevailing genetic diversity of *PvdbpII* gene and ensuing antibody mediated strain specific immunity and to in the context of low and unstable malaria conditions prevalent in two malaria endemic areas of Sri Lanka, and also from an area non-endemic to malaria.

Genetic diversity studies of the many of the vaccine candidate antigens of *P. vivax* malaria, including all previously published diversity studies on PvDBPII, used laboratory strain Salvador I (Ntumngia *et al.* 2009) as the reference. Thus, the present study too used the Sal-1 strain as the reference for analysis.

Isolates collected from the endemic area, Kataragama represented a parasite population restricted to a small area, consisting of seven contiguous villages, spanning an area of only 10 km². This certainly limited the detection of parasite populations that circulated in malaria patients in that region. In contrast, isolates collected from Anuradhapura, represented patients from the entire district. Further, the origin of parasite isolates collected from the NHSL Colombo, were traced to 11 different administrative districts of the island with different malarial transmission intensities throughout Sri Lanka. Therefore isolates from residents of Colombo possibly reflected a more comprehensive picture of the circulating parasite population in Sri Lanka, compared with those isolates collected from Kataragama and Anuradhapura.

Sequence analysis *PvdbpII* gene showed that only SNPs occurred in this antigen, confirming previously published sequences of global isolates, which is responsible for creating different alleles in the progeny. Conservation of the key DA contact residues and cysteins among the local PvDBPII sequences further confirmed that structurally and functionally important residues of the PvDBPII are conserved among different parasite populations globally (Chitnis and Sharma, 2007). Although some structurally and functionally important residues are

conserved within Minimal Receptor Binding Region (MRBR), most of the polymorphic residues of the PvDBPII molecule are also concentrated within this region (Tsuboi *et al.* 1994; Ampudia *et al.* 1996; Cole-Tobian *et al.* 2002; Ntumngia *et al.* 2009). Among the Sri Lankan isolates ~8% of the residues within the central 170 amino acid MRBR were polymorphic. Further, of the 21 total recorded polymorphisms identified from the present study, 61%, in which most were non-synonymous (dN), were concentrated within the MRBR. Higher numbers of dN mutations concentrated within the small area suggest that these variations are basically acquired as a mechanism of immune evasion. Similar notions on the role of polymorphism associated with the MRBR of the PvDBPII were previously reported for the parasite isolates collected from Papua new Guinea (PNG) (Cole-Tobian *et al.* 2002). According to the predicted Pv / Pkα DBL domain crystal structure, within the 3D intact molecule, most of the polymorphic residues were concentrated in two areas opposite to the DA binding site (Singh *et al.* 2006). However, of the total polymorphic residues in Sri Lankan isolates, only 48% were found in the predicted two polymorphic areas, while only 20% residues of these two regions were polymorphic. Similar types of distributions of the polymorphic residues within the intact 3D structure of the PvDBL domain were previously recorded globally for most of the published sequence data (Cole-Tobian and King, 2003; Tsuboi *et al.* 1994; Ampudia *et al.* 1996; Xainli *et al.* 2000; Kho *et al.* 2001). Thus these findings demonstrated that naturally occurring polymorphisms of the PvDBL domain are more extensive and widely dispersed over the DBL domain than predicted by Singh *et al.* (2006). In addition to those polymorphic residues lying within two polymorphic clusters, remaining isolated polymorphic residues were scattered over the entire 3D structure.

Only 4% of the nucleotides of the *PvdbpII* gene were polymorphic in Sri Lankan isolates. This level of polymorphism was comparatively lower than those recorded from PNG (10%) (Tusobi *et al.* 1994), but higher than in Colombia (2%) (Ampudia *et al.* 1996) and comparatively similar to Thailand (4%) (Gosi *et al.* 2008) and South Korea (5%) (Kho *et al.* 2001). Further, the 74% of the polymorphic sites with their corresponding nucleotides found in the present study were previously recorded from other different geographical malaria endemic regions, provide evidences that similar type of SNPs of *PvdbpII* genes are distributed throughout the world.

Of the 27 recorded SNPs of local isolates, 81% were dN in nature and these polymorphisms were located at all three base positions of different codons. This created a high level of microheterogeneity in the PvDBP antigen sequences among the Sri Lankan isolates. Thus the

39 nucleotide haplotypes resulted 33 different *PvdbpII* amino acid haplotypes / strains among the local isolates. Therefore, the circulating allelic repertoire observed in the current study had a greater diversity even under the prevailing low transmission and unstable malaria conditions in the island. These results were in agreement with the general inference that proteins involved in parasite recognition by the host's immune system are under strong selection pressure for accumulating polymorphism as a strategy for evading the host's immune attack (Escalante *et al.* 1998).

The distribution of the 3 most commonly occurring allele types within different districts of Sri Lanka suggested that no specific geographical isolations (with respect to different districts) occurred within the country. This was further strengthened by the finding that two most commonly occurring PvDBPII alleles in Sri Lanka were also found in South India, providing clear indication that Sri Lanka and India, two neighbouring countries, shared at least some common alleles of the *PvdbpII* gene.

VanBuskirk *et al.* (2004 a,) using alanine scan site directed mutagenesis demonstrated that 20 residues are involved in the DA binding site or play a functional critical role in receptor recognition, since the mutations of these residues completely disrupt the PvDBP binding to DA receptor. Most of these residues were mapped to discontinuous multiple locations on the surface of the DBL structure outside of the proposed binding site (reviewed by McHenry and Adams, 2007). The dispersed pattern of these functionally important residues on the surface of the DBL domain suggests some involvement in recognition / binding of the DA receptor or in subsequent molecular changes or interactions that stabilize the ligand–receptor complex (reviewed by McHenry and Adams, 2007). Of these 20 amino acids, only a single surface exposed residue (F306L) was found to be polymorphic among the field isolates in Sri Lanka. However, this polymorphism was only a restricted type (Phe to Leu) since the biochemical nature of the two amino acids were the same (both residues were non-polar). However, since residue at position 306 is not directly involved in receptor recognition according to the recently published crystal structure, and as this variation does not alter the biochemical nature of the amino acid type, this type of naturally occurring polymorphism is possible in amino acids lying within the minimum DA receptor binding region, mainly as a mechanism of evading antibody mediated immune mechanisms. This observation was confirmed previously among the field isolates from PNG, having a single polymorphic residue (N375D) with restricted variation (VanBuskirk *et al.* 2004 a). These findings of the field isolates from Sri Lanka and PNG were incompatible with previous suggestions that naturally occurring

polymorphisms within PvDBPII and the homologous *P. falciparum* EBA175 DBL domains generally do not alter erythrocyte-binding function of these two ligands (Xainli *et al.* 2000). However, compared to the significant effect of polymorphisms on receptor recognition in PvDBPII and PfEBA-175, it was previously demonstrated that allelic mutations in the DBL ligand domains of the *P. falciparum* paralogous BAEBL and JESEBL can alter their receptor recognition properties (Mayer *et al.* 2004). Since DBL domains of BAEBL and JESEBL can use multiple RBC surface receptors for invasion, each single amino acid change in the DBL domain of BAEBL and JESEBL leads to recognition of different erythrocyte surface receptors (VanBuskirk *et al.* 2004 a). As PvDBPII does not use alternative invasion pathways, this type of polymorphisms within the ligand domain does not exist naturally.

Although polymorphisms associated with the *PvDBPII* ligand domain do not seem to alter the functional ability to recognition of the erythrocyte DA receptor, VanBuskirk *et al.* (2004 b), demonstrated that few commonly existing allelic polymorphisms of PvDBPII molecule can change the antigenic character of the molecule. Three major polymorphisms described as directly involved in resistance to antibody inhibition of erythrocyte binding were W437R, I503K and N17K in minor intensity (VanBuskirk *et al.* 2004 b). Of these variants, N417K and W437R were found in <50% isolates while variant I503K were found at comparatively high frequencies (55%) in Sri Lankan parasite population. However, the frequencies of these three variant residues among the field parasite isolates from different geographical areas differed (VanBuskirk *et al.* 2004 b, Sousa *et al.* 2006, Gosi *et al.* 2008). The strong association found between two variants N417/W437 and K417/R437 among the Sri Lankan isolates were previously recorded for PNG (VanBuskirk *et al.* 2004 b) and Brazilian (Sousa *et al.* 2006) isolates too. Together with their functional effects of antibody inhibition of erythrocyte binding, these results indicate that residues 417 and 437 may constitute a discontinuous epitope in PvDBPII involved in immune evasion (Sousa *et al.* 2006). Importantly, it was further revealed that different combinations of these three polymorphic residues that confer highest as well as lowest resistance to binding inhibitory antibodies (VanBuskirk *et al.* 2004 b), were also prevalent among the Sri Lanka. Because the different combinations of polymorphisms associated with these three residues can alter the antigenic character without having a negative effect on its ligand function of the PvDBPII (causing significant changes in sensitivity to immune inhibition) (VanBuskirk *et al.* 2004 b), the presence of homologous polymorphisms within field parasite population suggest that positive selection acts on the *PvdbpII* gene, probably as the mechanism of immune evasion in Sri Lankan parasite isolates.

Two possible reasons may account for having dominant PvDBPII haplotypes (Cole-Tobian *et al.* 2007). First, certain dominant alleles may bind better to DA which may increase the fitness of the parasite. Second, several dominant PvDBPII haplotypes may be less immunogenic and thereby escape the host immune response (Cole-Tobian and *et al.* 2007). However more extensive analysis with higher numbers of isolates will elucidate the reason for the prevalence of high frequencies of certain PvDBPII alleles.

Comparison of Sri Lankan and global isolates revealed that 85% of the amino acid polymorphisms found locally was previously recorded in different malaria endemic geographical areas worldwide. More interestingly most commonly variant amino acids of PvDBPII found in global isolates (Sousa *et al.* 2006) compared to the DBP Sal-1 sequence were found with similar high frequencies in local sequences too. However, when considering amino acid haplotypes among the global and local isolates, a total of 117 families with considerable differences were found. Highest percentage of PvDBPII haplotypes found in local parasite populations were previously recorded in Thailand followed by two other countries from Asia, PNG and South Korea. However, according to the present analysis, although a single Sri Lankan isolate shared 100% homology to laboratory reference strain, Salvador -1 (Sal-1) (which is a common sequence from South American countries such as Brazil), 17 amino acid sequences from Colombia did not have any of the haplotypes similar to Sri Lanka. These data may suggest that, overall a higher level of identity occurs among Asian isolates compared to those from South America. Indeed, Sousa *et al.* (2006) suggested that higher percentage of identity were recorded for *PvdbpII* gene sequences from South American isolates mainly from Colombia and Brazil.

Pair wise nucleotide diversity (π) observed in the *PvdbpII* gene in the Sri Lankan isolates ($\pi = 0.0098$) is comparable with other *PvdbpII* gene sequences obtained for global isolates (range from 0.00904 in Colombia to 0.01108 in Thailand). Surprisingly, the variation at the nucleotide level within *PvdbpII* was highest in endemic area, Anuradhapura, in Sri Lanka, and not in Kataragama or even in PNG, two sites with a higher level of *P. vivax* endemicity than Anuradhapura. This high level of diversity in Anuradhapura was reiterated in two of the other diversity indices, *i.e.* average number of pairwise nucleotide differences and average number of sequence diversity for amino acid with p distance. The reason for higher level of genetic diversity in Anuradhapura compared to areas with high malaria endemicity is unclear, but compared to global isolates, it may be due to differences in sampling and sequencing techniques used in previous publications (*e.g.* PCR errors). Low level of diversity in

Kataragama may be due to the restriction of the size of the sample collection area (isolates from Kataragama were collected from 10Km² area, while in Anuradhapura the area was significantly greater).

Overall nucleotide diversity values of *PvdbpII* in Sri Lankan isolates were relatively lower compared to most of the other merozoite surface proteins of *P. vivax*, particularly than *Pvmsp-3α* (0.04662) (Mascorro *et al.* 2005), *Pvmsp-3β* (0.1056) (Rayner *et al.* 2004) and *PvMSP-1 P42* (0.02184) (Pacheco *et al.* 2007). However this was higher than that of another apical organelle protein, *PvAMA-1* domain II ($\pi=0.006$) (Thakur *et al.* 2008), but lower than the entire *PvAMA-1* gene ($\pi=0.0174$). Further, our research group had previously reported the genetic diversity associated with *P. vivax ama-1* gene using 23 parasite isolates from Sri Lanka (Gunasekera *et al.* 2007), where most of these isolates were used for the present study too. Accordingly, nucleotide diversity for the domain II (which showed highest diversity) of *Pvama-1* gene was 0.0097, highly comparable to the value of that of *PvDBPII*. Further this study when continued with a large number of isolates (N=96), the diversity for the entire *Pvama-1* gene was found to be 0.0095 (Chapter) even lower than *PvdbpII*. Further studies of the diversity of merozoites surface proteins of the same isolates revealed that nucleotide diversity of the *Pvmsp-1* gene ($\pi =0.0231$) (Chapter) was relatively high compared to the *PvdbII*, as recorded in other geographical areas. Low nucleotide diversity values of the *PvDBPII*, particularly than merozoite surface proteins may reflect a major functional restriction in the DBP protein, due to its crucial role in the invasion process (Martinez *et al.* 2004).

Isolates from the present study showed higher number of parsimony informative (Pi) sites compared to singleton variable (Si) sites. Further, the present analysis of global isolates revealed that this trend was found in Colombia and Thailand, while those from PNG and South Korea showed the opposite tendency. A high proportion of Si indicated a disperse substitution pattern, possibly searching for greater sequence diversity (for immune evasion or intraspecific competence) (Martinez *et al.* 2004). Thus, diversity generation may play a vital role in PNG and S. Korean isolates than in Colombian, Thailand and Sri Lankan isolates. On the other hand a more localized variation pattern (estimated by the proportion of Pi's) may be a response to a different type of pressure, acting in a more focused manner on specific sectors of the molecule, as observed in Colombia, Thailand (Martinez *et al.* 2004), and in Sri Lankan isolates.

In order to test for the selection pressure applied to the *PvdbpII* gene among the Sri Lankan isolates several different statistical tests were used, which complement and re-enforce each other, giving strongly consistent results (Escalante *et al.* 2004). The results of the population genetics analyses on the overall sequences gave evidence for existing, relatively strong selection pressure acting on the *PvdbpII* gene.

In the 100 sequences analysed in the present study, 85% of the polymorphisms were dN in nature that resulted in significant large excess of dN verses dS substitutions, observed in overall isolates as well as isolates from the different study areas indicating the evidence for natural selection. Although not statistically significant values for the *Z* test, the positive value for dN-dS (thus also resulted $dN / dS > 1$) gave evidence for the positive natural selection acting on *PvdbpII* gene in Sri Lanka. This tendency to present more dN than dS substitutions was previously observed in all other reports irrespective of the geographical locations (Ampudia *et al.* 1996; Tsuboi *et al.* 1994; Xainli *et al.* 2000; Cole-Tobian and King, 2003; Martinez *et al.* 2004). For isolates from different geographical areas examined, the ratio between dN / dS for *PvdbpII* ranged from 1.9 to 3.4 (Cole Tobian and King, 2003). Further, analysis of global isolates in the present study also resulted in a similar trend.

The MK test *P. knowlesi dbpa* gene was selected as an out group for comparison, due to being one of the most phylogenetically closely related species to *P. vivax*, since phylogenetically less related species are not useful for comparison. Statistically significant ($P < 0.05$) excess dN polymorphism within *P. vivax* compared to the divergence with its *P. knowlesi* DBP α orthologue were recorded for all 100 sequences and for sequences from malaria endemic area Anuradhapura. For sequences from Kataragama and non endemic Colombo similar type of results were obtained although the values for neutrality indices were not statistically significant. These data provide clear indication for significant departure from neutrality and indicated that purifying selection occurs in this region of the *PvdbpII* gene to reduce the number of dN substitutions between species. Results indicating that purifying selection is occurring in *PvdbpII* gene was also recorded from isolates collected from different geographical regions of the world (Cole-Tobian and King, 2003).

For isolates collected from malaria endemic area Anuradhapura, and non endemic area Colombo, Tajima's *D*, Fu and Li's *D* and *F* test for *PvdbpII* was positive but not significantly different from zero. A significant positive value for Tajima's test is evidence of possible balancing selection or population sub division, while a significant negative value suggests

recent directional selection, purifying selection or a population bottle neck (Tajima, 1989). Thus the positive value obtained for both statistics in local isolates indicate that *PvdbpII* alleles occur at more intermediate frequencies than expected with few alleles being rare or near to fixation. Such an observation is consistent with the action of balancing selection maintaining the allelic variation in the population (Baum *et al.* 2003). Thus, these results further strengthen the outcome obtained from MK test. However, the non significant negative value for Kataragama isolates, suggests that selective pressure in the form of negative selection acts on the *PvdbpII* gene in this area. Though not significant parasite isolates from Kataragama showed different selective pressures (positive / balancing and negative) according to the test used. Cole-Tobian and King (2003) recorded the same type of tendency showing both positive and negative selection pressure for parasite isolates collected from different malaria endemic regions. Martinez *et al.* (2004) suggested that this two contrasting selection pressures may imply a balance between a high functional restriction for maintaining structural constraint due to PvDBP's key role in the erythrocyte invasion process and search for diversity as a response against host immune system pressure as occurring in other *Plasmodium* surface antigens.

Recombination was also likely to contribute to maintain the observed diversity of *PvdbpII* gene among the Sri Lankan isolates. Comparatively low level of recombination events were recorded for overall isolates analysis as well as isolates from the three test areas, separately. However, even under the short distance of 672bp analysed, some of the polymorphic sites near each other on the *PvdbpII* gene showed LD, and this frequency of LD also showed significant decline with increasing molecular map distance. Both parameters evidenced mitotic recombination occurring within this antigenic locus among isolates collected from hypoendemic setting in Sri Lanka (Mendis *et al.* 1990). Because the diploid phase of the parasite life cycle takes place in the mosquito midgut, it is expected that sexual outcrossing and recombination is strongly associated with the intensity of malarial transmission (Gunasekera *et al.* 2007). Generally, levels of malaria transmission have a negative correlation with levels of LD (Anderson *et al.* 2000; Machado *et al.* 2004; Anthony *et al.* 2005). In areas where malaria transmission is high, levels of LD are likely to decrease because a mosquito can frequently acquire more than one genetically different clones in a single blood meal. Then, meiotic recombination of the parasite genomes will occur in the mosquito midgut and decrease the levels of LD. In contrast, in areas where malaria transmission is low, LD levels are likely to increase because, generally, the frequency of MCI and the number of clones per patient are lower than those in high transmission areas, and the chances of

acquiring more than one genetically different clone with a single blood feed by a mosquito will also be lower than those in high transmission areas. The results of this study may indicate that rare recombinant haplotypes generated within the Sri Lankan population are highly selectively advantageous or that new haplotypes generated by recombination in areas of higher endemicity sweep into the Sri Lankan population. If PvDBPII is subjected to balancing selection, recombinant PvDBPII haplotypes would be expected to accumulate in the worldwide population over long periods of time, and their diffusion into hypoendemic regions would bring the benefits of outcrossing to largely clonal populations (Gunasekera *et al.* 2007). Alternatively, these results may be related to the unique biological features of *P. vivax* infections, such as earlier gametogenesis and relapses, which increase the chance of mixed strain infections and sexual outcrossing, even in areas of low endemicity (Gunasekera *et al.* 2007). Recent use of PCR demonstrates that many individuals in malaria hyper endemic areas have chronic, asymptomatic infections often consisting of multiple parasite strains (Cole-Tobian *et al.* 2007). According to the present analysis approximately 18% of infections in the Sri Lanka population were multiple clone infections (MCIs) in nature.

Although evidence was apparent from the present study for meiotic recombination within the *PvdbpII* gene among Sri Lankan isolates, Cole-Tobian and King (2003), reported that recombination, does not appear to be an important factor contributing to the diversity of the *dbp* gene among isolates collected from Papua New Guinea, Korea and Colombia. However these authors suggested that two main factors, *i.e.* the tests used to detect the recombination (which may not be sufficiently powered to detect a low recombination rate), and also suggests that the *Pvdbp* gene may contain a mutational “hot spot” that could mask the presence of recombination in the *PvdbpII*, as possible reasons for not detecting the recombination among the isolates collected from malaria hyper endemic areas such as PNG (Cole-Tobian and King, 2003).

Compared to the *PvdbpII* gene, two of the other leading asexual erythrocytic stage vaccine candidate antigens, *Pvmsp-I* and *Pvama-1*, showed clear evidence for intragenic recombination in isolates collected from different geographical areas. Our research group previously reported (Gunasekera *et al.* 2007) that for the *Pvama-1* gene, recombination plays an important role in generating haplotype diversity (with 9 recombination events, linkage disequilibrium parameters R^2 and D decline with increasing distance and both average values for R^2 and D significantly larger among sites <300bp apart compared to sites >300bp apart), for 27 isolates collected from Kataragama and Colombo (most of these isolates were used for

the present study too). Further, Premawansa and co workers (1993) reported that for *Pvmsp-1* gene, Sal-1, Belem strains and recombinant form of these two strains were present in Sri Lankan isolates collected from malaria patients from the malaria non endemic area, Colombo. Evidence for recombination was also reported for EBA-175 region II, homologues gene for *PvdbpII* gene in *P. falciparum* (Baum *et al.* 2003). The results of the present study suggests that recombination acts as one of the events responsible for generating genetic diversity among Sri Lankan *P. vivax* population (at least for merozoite proteins), even under low and unstable malaria conditions prevalent in the country.

Between population divergence of the PvDBPII data in the present study (including a comparison between two endemic populations within Sri Lanka) and Sri Lanka as a whole with previously published PvDBPII data from global isolates were determined using θ estimator of Wright's fixation index (*Fst*) to determine the relative contribution of the differences observed between each population to the overall diversity observed using DnaSP ver 4.0 software. The *Fst* value ranged from 0 to 1, in which a value of 0 indicates that all alleles are evenly distributed among populations and value 1 denotes that an allele is found in only one population. Lower *Fst* values (0.1-0.15) indicate moderate genetic differentiation between populations while high *Fst* values (>0.15) suggest marked genetic subdivisions of populations (Conway *et al.* 2000). The *Fst* value between two endemic populations Anuradhapura and Kataragama within Sri Lanka (0.103), categorized as a moderate value, suggested that two distinct parasite populations existed between these two endemic areas. However, due to the low malaria transmission prevalent and geographical structure in Sri Lanka, it is difficult to explain the existence of such genetic differentiation between two endemic areas within the country, which are separated by a relatively low geographic distance. The restriction of the sampling area in Kataragama (only 10 Km²) may be one of the reasons for this high genetic differentiation between the two test areas.

Considering the overall results, the existence of relatively low genetic diversity, with marked evidences for the presence of LD, relatively low MCIs (18%), and the presence of relatively high population differentiation within endemic areas, reflected at *PvdbpII* sequences in the *P. vivax* population in Sri Lanka, is a probable reflector of the low level of malaria transmission prevalent in the island. Anderson *et al.* (2000), described the existence of a spectrum of population structures within a single pathogen species, *P. falciparum*. Accordingly, strong linkage disequilibrium, low genetic diversity, and extensive population differentiation are observed in regions of low malaria transmission, while random association among loci, high

genetic diversity and least geographical differentiation are observed in regions with intense malaria transmission. Thus, as described, the data from the present study are clearly in accordance with the population structure described in the areas of low malaria transmission. However, a recent study describing the microsatellite diversity of *P. vivax* in Sri Lanka, demonstrated evidence that deviate from the expected pattern from areas with low transmission: a significant multilocus LD co-occurring with extensive haplotype diversity and high proportion of MCIs was revealed (Karunaweera *et al.* 2008).

A phylogenetic tree of Sri Lankan and worldwide sequences did not support marked evidence towards geographical clustering of isolates. Sri Lankan isolates were distributed throughout the cladogramme with worldwide sequences. The observations derived from the phylogeny suggested that Sri Lankan and Thailand sequences were closely related and may appear to share a common ancestor (groups 7 and 9 exclusively contained isolates from Sri Lanka and Thailand). However, the overall observations suggested that despite the barrier to gene flow imposed mainly due to its island status, Sri Lankan sequences appeared to represent a sample from worldwide genetic diversity rather than from any particular lineage of worldwide *PvdbpII* sequences.

Additionally, these results demonstrated that although there are some differences and similarities that exist in the nucleotide and amino acid variation in PvDBP_{II} among isolates from Sri Lanka, PNG, Thailand, South Korea and Colombia, more importantly, unique variants from Sri Lanka do exist and should be considered in future vaccine development of PvDBP_{II}. Two recently concluded studies, describing the genetic diversity of AMA-1 (Gunasekera *et al.* 2007) and microsatellite diversity (Karunaweera *et al.* 2008) of *P. vivax* from Sri Lankan isolates, also demonstrated that no significant geographic clustering was evident in *P. vivax* population in Sri Lanka, compared to worldwide *P. vivax* populations. Further consistence with our results, mitochondrial DNA haplotypes did not show clustering into continents in a recent large scale study of field isolates and monkey-adapted strains of *P. vivax* (Mu *et al.* 2005). The present highly diverse phylogenetic tree was constructed relying on polymorphisms based on a single gene, the *PvdbpII*, demonstrating the high degree of topological variation that can be found using this technique, but also highlighting the problems that can be associated with gene sampling in phylogenetic studies (Gosi *et al.* 2008). Use of a combination of several genes with different rates of evolutions has been suggested as an efficient way to overcome this difficulty to prove the existence of a unique tree relating these sequences (Gosi *et al.* 2008).

Identification and characterization of potential B and T-cell epitopes within vaccine candidate antigens is important to evaluate the candidacy of that molecule. Polymorphic residues that cluster in areas immunodominant for B and T-cells of the molecules may have arisen to reduce the host immune pressure, by altering the immune response to these variant amino acids. Of the described five each of B and T cell epitopes, only 2 from each category contained polymorphisms in Sri Lanka. However the number of epitopes that contained polymorphic residues in Sri Lankan isolates were comparatively low than that of global isolates which showed each of these peptides contained polymorphic residues (Cole-Tobian *et al.* 2003). However, similar to global condition all the observed polymorphisms in Sri Lankan sequences were non-synonymous in nature creating high level of genetic diversity. Thus as described it can be suggested that these results are consistent with the hypothesis that host immunity to PvDBPII contributes to greater diversity via natural selection. Of the two universal epitopes of PvDBPII described (Saravia *et al.* 2008), peptide ISVKNAEKVQTAGI VTPYDI contain polymorphic residues in isolates from Sri Lanka and from different geographical areas (Table 4.5). Although the other predicted epitope is conserved among the Sri Lankan isolates, this also contains polymorphic residues in isolates from PNG and South Korea. These results suggested that the description of the two peptides as universal PvDBPII epitopes may not be applicable in certain areas where malaria is endemic.

Polymorphism in T-cell epitope regions of parasite antigens may well enable parasites to escape host immune responses, as polymorphism in T-cell epitopes can up or down regulate a T cell response to the index peptide, or completely arrest an immune response assisting the parasite escape the host immune system (Tanabe *et al.* 2007). Thus, CD4⁺ T helper cells play important role both in humoral and cell mediated arms of the immune system. As T-helper cell activation depends on antigen present in contact with HLA class II molecules and HLA is the most polymorphic genes found in the human genome, the prediction which antigenic fragments can bind to HLA-II molecules is an important task in identification of CD4⁺ T cell epitopes. In developing a protective immune response against protein antigens, CD4⁺ T helper cells play important role both in humoral and cell mediated arms of the immune system. In humans, HLA-DRB is the most prominent HLA-II found among the surface of the antigen presenting cells. However, since HLA is the most polymorphic genes found in the human population, the expressed HLA-DRB type on the surface of an antigen presenting cells is also a key factor in deciding the different types of the epitopes expressed to T cells to initiate the T cell dependent immune response. Using epitope prediction software SYFPEITHI, PvDBPII

Sal-1 strain epitopes expressed by the HLA-DRB-1 alleles were identified. In SYFPEITHI software epitope prediction was based on published motifs (pool sequencing, natural ligands) and taking into consideration the amino acids in the anchor and auxiliary anchor positions, as well as other frequent amino acids. Further, considering the T-cell epitopes only those which are likely to be naturally processed have been selected in this software (Rammensee *et al.* 1999). A scoring system, which evaluates the every amino acid within a given peptide, depending on whether they are anchor, auxiliary anchor or preferred residue, are also considered and summation is given as a score which can be used as an indicator of the binding capacity of a particular epitope (Rammensee *et al.* 1999). Only those with a binding score equal to or more than 25 were predicted as enabling effective binding and were considered as effective epitopes. Of the different HLA-DRB-1 alleles, only four were selected, since these four were among the 10 most prominent human HLA-DRB-1 alleles in Afroamerican and Asian populations, where malaria is endemic (Southwood *et al.* 1998). According to the results, SYFPEITHI software predicted all four HLA-DRB-1 alleles to be able to bind and express PvDBPII Sal-1 peptides with a binding score ranging from 25 to 30. The binding of different numbers of peptides of PvDBPII Sal-1 strain suggested that HLA-DRB-1 alleles expressed different binding profiles. Some of the polymorphisms associated with the predicted PvDBPII peptides in Sri Lankan *P. vivax* population was able to change the binding and expression capacity of that particular epitope, and thus change its ability to be expressed as a T-cell epitope. Since most of these polymorphisms can reduce the binding capacity of that particular epitopes to the HLA-DRB molecules it can be suggested that such polymorphism arose as a mechanism to evade the host immune response. However, since the confidence level for predicting epitopes to MHC class II molecules is not usually very high (approximately 50%), and because these molecules accept a wider range of peptides in size and binding registers, the predicted results must interpreted with caution. Although the use of bioinformatics tools for the prediction of epitopes are useful, care must be taken always to confirm the predicted results with experimental results. For example, a recent study by Patarroyo and coworkers (2008), showed data obtained from experimental studies for HLA-DRB alleles that agreed with the epitopes predicted by the SYFPEITHI software while some did not contain a predicted epitope but had strong binding with those alleles immunologically.

Malaria is known to be a heterogeneous disease caused by many parasite strains / subpopulations. It is known that a limited set of immunodominant polymorphic antigenic determinants elicit life-long responses associated with the early acquisition of immunity to malaria, while weaker responses to conserved antigens are probably involved in the later

development of the anti-parasite immunity. Therefore, an effective vaccination strategy for malaria would be based on polymorphic antigens, the composition which should be changed regularly to match locally prevalent antigenic variants.

Strain specific malaria immunity defines protection in terms of the ability of generating effective immune responses against the antigenic variants to which individuals are locally exposed. Most of the early studies with repeated infections using homologous and heterologous strains in humans have suggested that the immunity raised against *P. falciparum* is strain dependant (Jeffery, 1966). Later on ample evidence for the existence of strain dependence antibody responses as well as strain dependence immunity was reported, based on the experimental evidence and field base studies. For example, in nonhuman primates, it was demonstrated that immune sera from aotus monkeys contained antibodies that blocked or reversed cytoadherence *in vitro* and that were isolate specific (reviewed by Doolan *et al.* 2009). In field base experiment, *P. falciparum* Merzoite Surface Protein-1 targeted by naturally acquired antibody responses were predominantly type specific and correlated with the parasite genotype present at the time of infection clearly implicating the presence of strain specific immunity (Cavanagh *et al.*, 1998; Da Silveira *et al.*, 1999). In a pilot study carried out in Sri Lanka on strain specific immunity of *P. vivax* MSP-1 C-terminal hypervariable domain, it was revealed that immune responses correlated with the homology of the allele in their current infection (Manamperi, 2003). Strain specificity of antibody recognition and growth-inhibitory activity has been mostly documented for one of the leading blood-stage vaccine candidate antigens, *P. falciparum* AMA-1 (Coley *et al.* 2006; Healer *et al.* 2004; Hodder *et al.* 2001; Kennedy *et al.* 2002, Miura *et al.* 2007).

Despite ample evidence for the strain dependence of the immune responses to malaria, there is evidence, both *in vitro* and *in vivo*, that naturally acquired immunity against malaria contain both strain specific as well as strain transcending arms of immunity (reviewed by Doolan *et al.* 2009). Although most of the experimental data revealed that protective immunity against heterologous challenge with distinct strains of *P. vivax*, *P. cynamolgi* and *P. falciparum* was less effective than that against homologous strains and degree of protective immunity induced by malariatherapy against challenge with heterologous strains was almost always less effective than that against homologous challenge, these experiments suggested that there is a conspicuous partial protection with homologous immunity to heterologous challenge (reviewed by Doolan *et al.* 2009). Further, the presence of cross reactive antibodies against

Variant Surface Antigens of *P. falciparum* infections among the adults in an area of hyperendemicity in India was demonstrated (Chattopadhyay *et al.* 2003).

Evidence for the existence of naturally acquired strain specific as well as transcending / cross reactive anti-PvDBPII antibodies was found in the present study. Indications for the presence of strain specific antibody responses were obtained from the existing positive relationship between the total (IgM+IgG) antibody magnitudes / levels with the frequencies of the six of the most common PvDBPII amino acid haplotypes. Similar type of observation was recorded recently in high malaria transmission area in Papua New Guinea where the antibody levels positively correlated with the relative frequencies of the most common PvDBPII haplotypes (Cole-Tobian *et al.* 2009). Thus, these findings clearly support evidence for the existence of strain specific naturally acquired anti-PvDBPII antibodies. Except for only three strain types (SL -19, 22 and 30), all other PvDBPII strains recorded in the present study contained naturally acquired total (IgM+IgG) antibodies, which bind PvRII, a recombinant protein based on PvDBP Sal-1 strain. This provides evidence for the presence of cross reactive antibodies that at least reacted against two different PvDBPII strains. This evidence was further strengthened by the presence of naturally acquired cross reactive functionally important anti-PvRII IgG antibodies (except for strain types SL-11, 16, 21, 25 and 30). Primarily, due to the low malaria transmission existing in Sri Lanka which result in comparatively low level of re-infection, it can be suggested that the naturally acquired anti-PvDBPII antibody responses measured in the present study, were basically directed against the PvDBPII strain present in the current *P. vivax* infection of that patient, rather than antibodies directed against previous infection/s. This suggestion was further reiterated by the long term acceptable assumption that in malaria repeated exposure is required for the maintenance of the naturally acquired immune responses.

As PvDBPII is highly polymorphic, it is important to find out whether PvDBPII specific binding inhibitory antibodies recognize naturally existing PvDBPII variants and whether receptor-binding inhibition is strain dependent or independent. It was demonstrated that, among the naturally acquired binding inhibitory antibodies produced against 26 locally circulating PvDBPII SL strains, 62% showed cross reactive inhibition against the Sal-1 strain. Although the frequency of the PvDBPII Sal-1 among the local circulating *P. vivax* strains was restricted (only 1%), presence of naturally acquired cross reactive binding inhibitory antibodies (between Sal-1 and locally prevalent PvDBPII strains) is an important observation, since many of the vaccine candidates against *P. vivax* malaria (including PvDBPII) are based

on sequences from the laboratory maintained strain Sal I (Ntumngia *et al.* 2009). Importantly these results indirectly support evidence for the notion that a vaccine based on PvDBPII Sal-1 strain may be effective against most of the diverse PvDBPII strains circulating under low malaria transmission condition in Sri Lanka. King *et al.* (2008), recently showed that, in a cohort of Papua New Guinean children, naturally acquired antibodies with high level of binding inhibitory activity (>90% inhibition) showed strain transcending / cross reactive inhibitory activity by reducing re-infection with similar efficiency of Papua New Guinean *P. vivax* strains characterized by six diverse PvDBPII haplotypes, where a majority of serum samples with antibodies having intermediate (50-90%) and low level (>50%) of inhibition showed strain specific inhibitory response. Subsequently it was demonstrated that PvDBPII specific antibodies are associated with strain specific immunity in the same cohort of children, where high levels of naturally acquired antibodies against three most common PvDBPII variants showed a delay in the time taken to re-infection with homologous than heterologous parasite strains and also showed lower incidence density parasitaemia (Cole-Tobian *et al.* 2009). However, they also showed that the protection engaged by the high level of binding inhibitory antibodies was not only strain specific but also strain transcending. These studies clearly indicates the significance of strain specific as well as strain transcending immune responses against an important invasion ligand of Duffy binding protein of *P. vivax*.

In conclusion, the data presented in this study allowed the characterization of the prevailing genetic diversity of the PvDBPII in parasite isolates from Sri Lanka, an island where unique low and unstable malaria transmission patterns exist. Although, of the 21 amino acid polymorphisms recorded in Sri Lankan isolates, only 3 seems to be unique to the country, the most prevalent PvDBPII strain type (SL-1) was also unique to Sri Lanka. Thus, with the level of sequence polymorphism associated with PvDBPII in local isolates, with the presence of unique PvDBPII strains in high frequencies within the country compared to other different malaria endemic geographical areas, suggest importance of the data generated which must be carefully pursued in planning future vaccine development based on PvDBP. The demonstration of the presence of strain transcending PvDBPII specific functionally active binding inhibitory antibodies among locally exposed *P. vivax* patients suggest that efficacy of a vaccine based on PvDBP Sal-1 may be high among the local population. Overall the results of the present study clearly provide baseline evidence that should be considered in future vaccination strategies based on PvDBPII, not only locally, but globally.

7. Conclusions

1. The prevailing genetic diversity at *Pvmmsp-3α*, the frequency of MCI and the association of particular genotypes with particular endemic areas highlight the complexity of the *P. vivax* infections in Sri Lanka.
2. Under low transmission and unstable malaria conditions prevalent in Sri Lanka, extensive allelic polymorphism was evident at *Pvmmsp-1₃₃* due to recombination, mutation and positive selection.
3. PvMSP-1₁₉ was conserved in all local clinical isolates, and the isotype switch of the anti-PvMSP-1₁₉ response from IgM to IgG with increasing exposure in endemic areas showed the protective nature of this antigen.
4. The genetic polymorphism of the domain II of *P. vivax ama-1* gene in Sri Lanka was due to single nucleotide polymorphisms (SNPs) and host immune pressure.
5. The highly conserved domain II loop of PvAMA-1 in all clinical isolates in Sri Lanka demonstrated the protective immune response by the marked isotype switch from IgM to IgG against peptide P04 on the domain II loop with increasing exposure in residents from endemic areas.
6. A relatively low level of allelic diversity exists among the *PvdbpII* gene in Sri Lanka and appears to be under positive selection pressure where meiotic recombination was found to be a significant factor in maintaining genetic polymorphism. *PvdbpII* phylogeny suggests that local *P. vivax* parasites represent a sample of the global population.
7. Polymorphism in T cell epitopes arising due to non-synonymous substitutions in PvMSP-1₃₃, PvAMA-1DII and PvDBPII may result in differential binding of the polymorphic peptides to class II MHC alleles, and may evoke different host immune responses.

8. The binding inhibition assay which analyzed the functional relevance of the naturally acquired antibodies revealed that approximately half of the *P. vivax* infected symptomatic patients developed anti-PvRII binding inhibitory antibodies. The comparatively low level of % parasitaemia and close relationship between the binding inhibitory antibodies with IgG1 and IgG3 cytophilic antibodies (previously recorded data) observed among the patients with high level of anti-PvDBP-II binding inhibitory antibodies indirectly indicates acquisition of “protection” against blood stage of *P. vivax* infections, even at the low and unstable malaria conditions prevalent in Sri Lanka.
9. PvDBP-II binding inhibitory antibodies with ability to functionally cross react with PvDBP-II Sal-1 reference strain. This suggests that efficacy of a vaccine based on PvDBP Sal-1 may be high among the local population.

Future Work:

1. To construct a multi component vaccine containing the PvMSP-1₁₉, P04 of PvAMA-1 domain II and PvDBP-II regions.
2. To examine the humoral response in primates following immunization with the above combined construct.

Problems if any, encountered during the implementation of the project:

We faced a problem with obtaining sequences of the malarial antigens. The first company that undertook this task did not produce any valid results. We then had to seek another company and then the samples had to be reprocessed and sent for sequencing. The outcome was satisfactory. However, this mishap led to losing 4-5 months of project time. Hence, an extension was requested from the NSF which was granted up until 30th of November, 2008.

Further, as the bioinformatics analysis of the sequences thus obtained were carried out in collaboration with Prof Ananais Escalante, Evolutionary Biologist of the Arizona State University, USA it took us an unexpectedly long time period to finalise this analysis for all three antigens involved in this study, by corresponding with him on email and through skype.

Therefore, regrettably we could not keep to the deadline for submission of this report in December, 2008.

8. Appendices

Appendix I - NET Buffer (pH 7.5)

5M NaCl	-	1.5 ml
0.5M EDTA	-	1.0 ml
1M Tris-HCl	-	2.5 ml
Distilled water	-	45 ml

50 ml of 1X NET buffer contains

150 mM NaCl

10 mM EDTA

50mM Tris-HCl

Appendix II - TE buffer (pH 8.0)

1M Tris-HCl	-	500 μ l
0.5M EDTA	-	100 μ l
Distilled water	-	up to 50 ml

50 ml of TE buffer contains

10 mM Tris-HCl
1 mM EDTA, pH 8.0
Distilled water

Appendix III - Phosphate-Buffered Saline (PBS), pH = 7.4

Stock solution – 100X PB, pH= 7.4

K_2HPO_4 - 43.55g (250mM)

Dissolved in distilled water and made the final volume up to 250ml (1mol/L).

KH_2PO_4 - 10.20g (7.50mM)

Dissolved in distilled water and made the final volume up to 75ml (1mol/L)

K_2HPO_4 solution titrated against KH_2PO_4 solution until pH becomes 7.4

Working solution - 1X PBS, pH= 7.4

100X PBS - 10 mL

NaCl - 29.22 g (1M)

Dissolved in distilled water and made the final volume up to 1000ml.

Appendix IV - Blocking Buffer

- a) 5% w/v non-fat milk powder (Anchor, New Zealand) in 1X PBS (pH=7.4)
- b) 2% w/v non-fat milk powder (Anchor, New Zealand) in 1X PBS.

Appendix V– Washing buffer

- a) 0.2% Tween 20 (Polyoxyethylenesorbitan monolaurate, Sigma, USA) in 1X PBS (pH=7.4).
- b) 0.05% Tween 20 (Polyoxyethylenesorbitan monolaurate, Sigma, USA) in 1X PBS (pH=7.4).

Appendix VI– Dilution Buffer

- a) 5%w/v non-fat milk powder (Anchor, New Zealand) in washing buffer (a).
- b) 2.5% w/v non-fat milk powder (Anchor, New Zealand) in washing buffer (b).

Appendix VII – Substrate Buffer

Citric acid - 5.92 g
Na₂HPO₄ (anhydrous) - 6.19 g
Dissolved in distilled water and made the final volume up to 1000ml.

Appendix VIII – Sulphuric Acid (2N)

Conc. H₂SO₄ - 15 ml
Mixed with distilled water and made the final volume up to 1000ml.

Appendix IX - Sodium bicarbonate buffer (pH 9.6)

Na₂CO₃ – 0.160 g
NaHCO₃ – 0.295g
Dissolved in sterile distilled water and made the final volume upto 100 ml.

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Section 4

Impact of Research results:

i) **Relevance of results achieved to scientific advancement –**

The final outcome of this study lead to the identification of three veritable vaccine components that could be included in a protective vaccine against vivax malaria in Sri Lanka.

ii) **Relevance of results achieved to national/socio-economic development –**

Based on this work, 2 postgraduate students have completed their doctoral theses submitted to the University of Colombo. International collaborations with the Harvard School of Public Health - USA, Institute Pasteur Paris, International Centre for Genetic Engineering and Biotechnology - New Delhi and Arizona State University – USA, further enhanced the calibre of the related human resource development which also included an immense opportunity for undergraduate and post graduate research training.

iii) **Dissemination/application of research output –**

This project has resulted in 19 research communications presented both at local and international forums. Further, one scientific paper was published in an international peer reviewed journal while two more are under the review process. Two more manuscripts are currently being finalised.

The outcome of this research will contribute much to improve the development of MSP-1, AMA-1 and DBP as vaccine candidates against the asexual erythrocytic stages of vivax malaria in the form of an antigen construct to be used in Phases I and II vaccine trials in Sri Lanka in the foreseeable future.

Section 5

Miscellaneous

- i) List of major equipment acquired during the project period and their functionality
– A desktop computer and a Laser printer
- ii) List of publications/communications arising from the project and/or presentations made at seminars, workshops etc. (Please see Annexure I)

Section 6

Summary Statement of Expenditure (indicate under Personnel, Equipment, Consumables, Travel and Subsistence and Miscellaneous)
(Please see Annexure II)

Section 7

- i) Grantees' signatures: *P. Randeniya*
- ii) Comments of the Head of the Department/signature: *May good outcome*
N. Pallewatte
- iii) Head of the Institution's signature: *[Signature]*
Vice-Chancellor
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associated with the prevailing pattern and intensity of transmission in the study area (Bruce *et al.*, 1999, 2000; Engelbrecht *et al.*, 2000; Magesa *et al.*, 2002). In areas where the entomological inoculation rate (EIR) is below one infectious bite/person-year, only 30%–60% of the human infections have been found to be MCI (Suwanabun *et al.*, 1994; Joshi *et al.*, 1997; Cui *et al.*, 2003). In areas of intense transmission, such as many parts of Papua New Guinea, however, >65% of the human malarial infections investigated have been identified as MCI (Kolakovich *et al.*, 1996; Bruce *et al.*, 2000; Cole-Tobian *et al.*, 2005).

The genotyping of *P. vivax* infections has been based on several polymorphic antigens (Premawansa *et al.*, 1993; Kolakovich *et al.*, 1996; Bruce *et al.*, 1999, 2000; Cui *et al.*, 2003; Cole-Tobian *et al.*, 2005). The gene coding for the parasite's merozoite surface protein-3 α (*msp-3 α*) appears to be a particularly reliable marker for the molecular epidemiology of *P. vivax* in various geographical areas, having a central polymorphic region that is readily evaluated by use of a combination of PCR and RFLP (Bruce *et al.*, 1999, 2000; Galinski *et al.*, 1999; Cui *et al.*, 2003; Cole-Tobian *et al.*, 2005; Véron *et al.*, 2009).

In Sri Lanka, human malaria is considered to be unstable, with generally low levels of transmission (Rajendram and Jayawickreme, 1951). Almost the entire dry zone of the country is considered to be endemic for *P. vivax* and this species accounts for 60%–80% of the total annual incidence of malaria in the country (Anon., 2000), inclusive of relapse infections (Fonseka and Mendis, 1987). The complexity of the *P. vivax* infections in Sri Lanka has previously been demonstrated in a few small-scale studies based on various markers and tools (Langsley *et al.*, 1988; Udagama *et al.*, 1990; Premawansa *et al.*, 1993; Gunasekera *et al.*, 2007; Karunaweera *et al.*, 2008; Manamperi *et al.*, 2008). In the present, relatively large-scale investigation, the genetic complexity of *P. vivax*, in

clinical isolates from Sri Lanka, was explored via the *msp-3 α* marker.

SUBJECTS AND METHODS

Ethics, Sample Collection and DNA Extraction

The study protocol was approved by the Ethics Review Committee of the University of Colombo, Sri Lanka. Blood samples were only collected with informed consent.

Between December 1998 and March 2000, pre-treatment blood samples were collected, in three areas (Fig. 1), from 217 adults (aged >15 years) with microscopically confirmed *P. vivax* infections. The subjects either came from the *P. vivax*-endemic areas of Anuradhapura (8°22'N, 80°20'E; N=62) and Kataragama–Buttala (6°25'N, 81°20'E; N=91) or lived in non-endemic Colombo (7°55'N, 79°50'E; N=64) but had been infected during visits to regions of the island with *P. vivax* transmission (Fonseka and Mendis, 1987). Age, gender, number of malarial attacks experienced, time symptomatic prior to treatment, and time since previous malarial attack were recorded for each subject.

Genomic parasite DNA was extracted from 1-ml samples of venous blood (Gunasekera *et al.*, 2007). Briefly, plasma and white blood cells were removed from the whole-blood samples, by centrifugation and filtration through a CF11 column, respectively. The erythrocytes eluted from the column were lysed with 0.015% saponin in NET buffer (150 mM NaCl, 10 mM EDTA and 50 mM Tris; pH 7.5), pelleted by centrifugation, and then treated with 1% (w/v) N-lauroyl-sarcosine, RNase A (100 μ g/ml) and proteinase K (200 μ g/ml). Parasite DNA was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1, by vol.) and precipitated with 0.3 M sodium acetate and absolute ethanol. The DNA pellets were air dried, resuspended in Tris buffer (10 mM Tris and 1 mM EDTA) and stored at –20°C until further use.

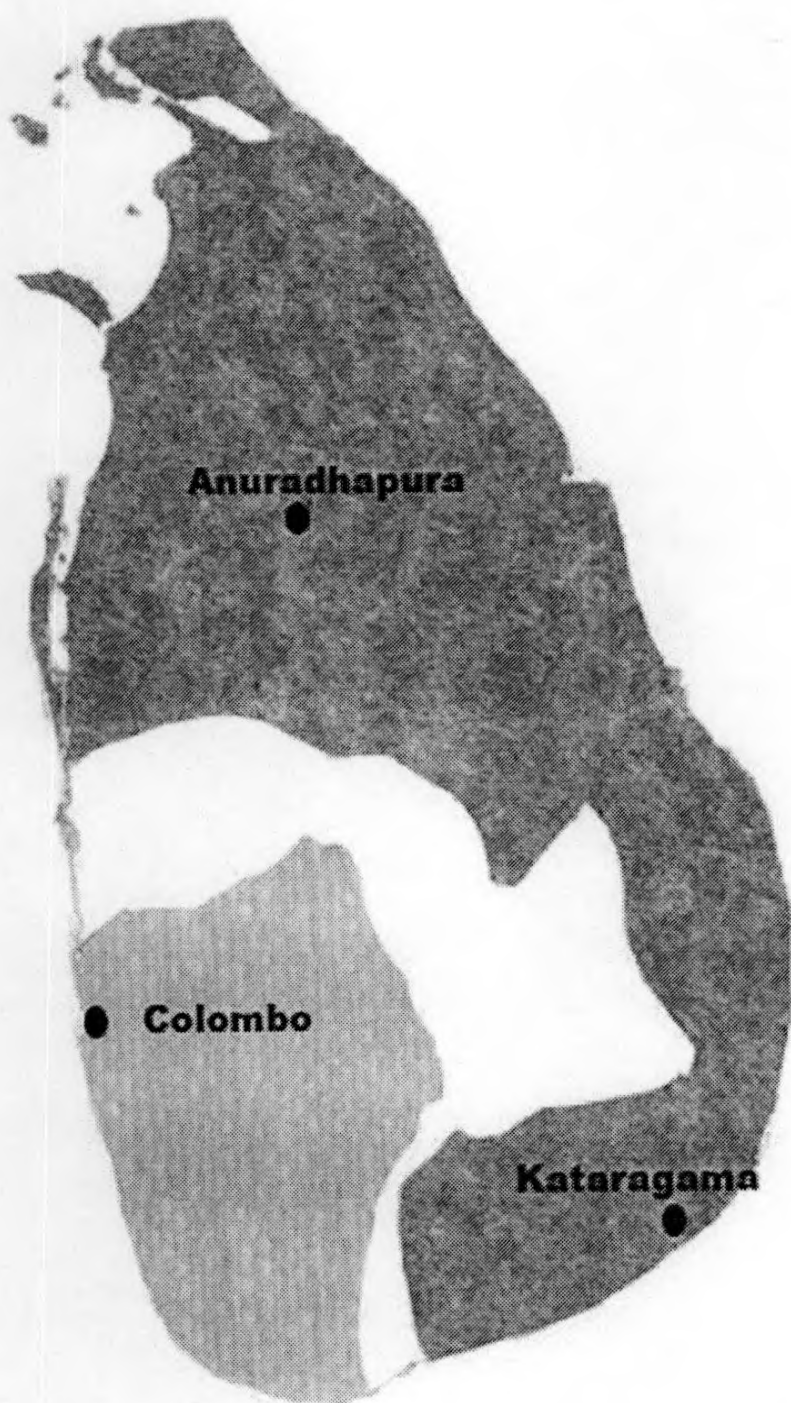


FIG. 1. Map of Sri Lanka showing the extent of the dry, malaria-endemic zone (■) the wet, non-endemic zone (■) and the intermediate zone (□), as well as the locations of the three sample-collection sites.

Parasite Genotyping and Identification of Single-clone Infections

The clonality of each *P. vivax* infection was investigated using PCR-RFLP analysis of alleles at the polymorphic *msp-3α* locus (Fig. 2) and a slight modification of the method described by Bruce *et al.* (1999). Nested PCR amplified a fragment (of approximately 1900 bp) of the polymorphic region of the *msp-3α* locus. For this, the P1 (5'-CAG CAG ACA CCA TTT AAG G-3') and P2 (5'-CCG TTT GTT GAT TAG TTG C-3') primers were used for the outer PCR while N1 (5'-GAC CAG TGT GAT ACC ATT AAC C-3') and N2 (5'-ATA CTG GTT CTT CGT CTT CAG G-3')

were used for the nested reaction. Each amplification was carried out in a 20-μl reaction mixture containing either 1 μl of DNA extract (primary round) or 0.5 μl of the products of the primary reaction (nested round). The final amplicons were subjected to digestion with 2.5 U *Hha*I (New England BioLabs, Ipswich, MA) or 5 U *Alu*I (New England BioLabs), in a reaction volume of 20 μl. Whichever enzyme was used, the reaction mixtures were incubated for 4–5 h at 37°C and then 20 min at 65°C.

After the digestion fragments produced in each reaction were separated by electrophoresis, the lengths of the DNA fragments (in bp) were estimated. Any MCI were identified either directly from the PCR, when more than one band was obtained from the sample, or from the RFLP analysis, when the sum of the lengths of the fragments produced from a digestion with *Hha*I or *Alu*I was greater than the size of the uncut PCR product. Isolates identified as single clones were chosen for further analysis.

DNA Sequencing and Alignment Analysis

The amplicons from the PCR were resolved on agarose gels and purified using a commercial kit (Promega, Madison, WI) before direct sequencing. Each amplicon was sequenced commercially three times, in both the forward and reverse directions, using the N1 and N2 primers again, a forward (5'-CAG TAG TGG CAA AGG AGG AAG-3') and reverse (5'-AAA TGC AGC AGA GTC AGC CA-3') internal primer, and BigDye® termination chemistry (Macrogen, Seoul), achieving six-fold sequence coverage. A single continuous sequence of approximately 1200–1900 nucleotides was derived from the isolates from 17 subjects with single-clone infections (GenBank accessions GU175269–GU175285). These sequences were aligned and verified using the Seqman II software package (DNASar, Madison, WI) while consensus sequences were aligned using the ClustalW program from version 4.1 of the

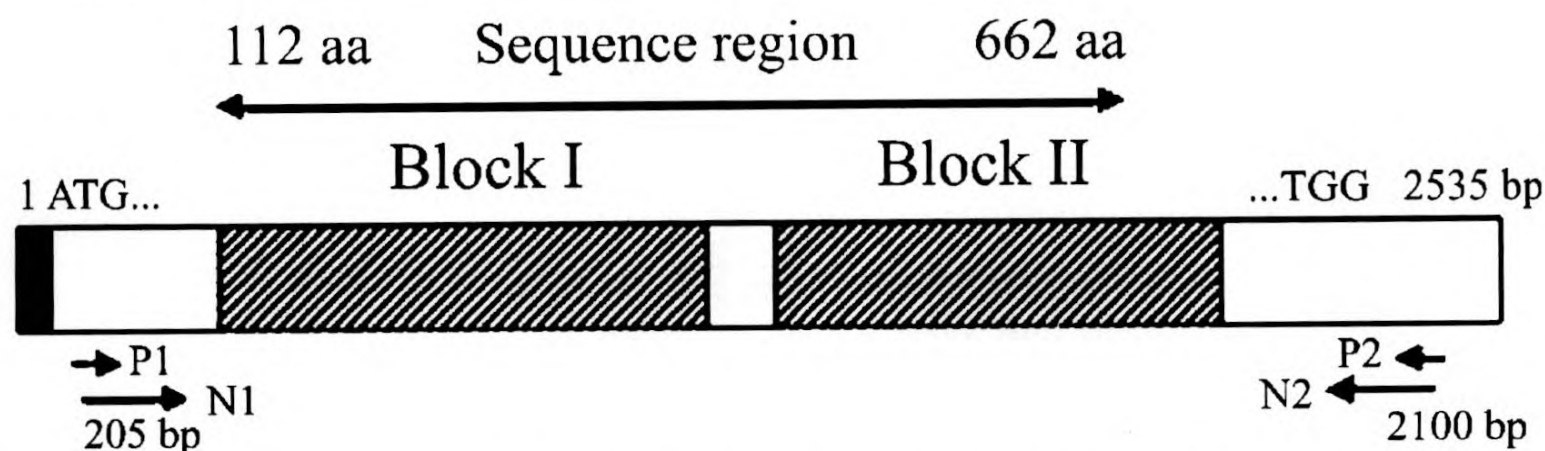


FIG. 2. Schematic representation of the *msp-3α* structure, showing the signal peptides (■) and the two blocks of coiled-coil structural domain in the polymorphic region (▨). The positions of the primary (P1 and P2) and nested (N1 and N2) oligonucleotide primers used in the PCR are indicated by arrows. Numbers refer to the nucleotide base pairs (bp) within the corresponding sequence of the Belem strain of *Plasmodium vivax* or the amino-acid (aa) positions in the readable sequence of the *msp-3α* gene in Sri Lankan isolates.

MEGA software package (Tamura *et al.*, 2007; www.megasoftware.net).

Various indices of genetic diversity — π (nucleotide diversity), k (mean number of nucleotide differences), S (number of polymorphic sites), H (number of haplotypes) and H_d (haplotype diversity) — were calculated using version 4.5 of the DnaSP software package (Rozas *et al.*, 2003), while synonymous and non-synonymous nucleotide diversities were evaluated using version 4.1 of the MEGA software package and the method of Nei and Gojobori, with the Jukes–Cantor correction (Tamura *et al.*, 2007). The MEGA software package was also used to produce a phylogenetic tree from the aligned nucleotide sequences, employing the neighbour-joining (NJ) method with 1000 bootstrap replicates and the Kimura two-parameter distance model.

Seasonal Malaria Prevalence and MCI in the Endemic Kataragama–Buttala Area

The Kataragama–Buttala area of the Monaragala district of Sri Lanka's Uva province has been endemic for *P. vivax* malaria for many years (Briët *et al.*, 2003). Although malaria is reported throughout the year in this area, the incidence of the disease peaks between November and late February, during the north-eastern monsoon rains. At the same time as the sample collection for the PCR–RFLP analysis, four

mass blood surveys were carried out in three adjacent villages in this area, at approximately 3-month intervals, between December 1998 and December 1999. The protocol for these surveys was also approved by the Ethics Review Committee of the University of Colombo, Sri Lanka. Stained thick and thin bloodsmears were used for the microscopic detection of malarial infection in the villagers. Attempts were then made to establish association(s) between the (rainfall-related) seasonal variation in the prevalence of *P. vivax* infection in the villagers and the occurrence of MCI in the Kataragama–Buttala area.

Statistical Analysis

The collected data were analysed using version 11 of the SPSS for Windows software package (SPSS Inc, Chicago, IL) and version 6 of the Epi Info package (Centers for Disease Control and Prevention, Atlanta, GA). Proportions of independent samples were compared using χ^2 test while means were compared using Student's *t*-tests and analysis of variance, as appropriate. A *P*-value of <0.05 was considered indicative of a statistically significant difference.

RESULTS

Characteristics of the Test Populations

Although $>90\%$ of the subjects from the endemic areas presented (for malaria

diagnosis and treatment) within 4 days of the onset of their symptoms, only 50% of the subjects from non-endemic Colombo had sought treatment within 6 days of becoming symptomatic. The mean ages of the subjects from the three study areas were similar ($P>0.05$ for each comparison) but the subjects from Kataragama-Buttala reported a significantly higher number of previous malarial attacks than the subjects from Anuradhapura or Colombo, with means of eight, three and two, respectively ($P<0.05$). The subjects from Anuradhapura, Kataragama-Buttala and Colombo presented with means of 0.10%, 0.07% and 0.14% of their erythrocytes infected, respectively, the values for Kataragama-Buttala and Colombo being significantly different ($P<0.05$). Although half of the subjects from Anuradhapura had had a malarial attack in the previous 5 months and half of those from Kataragama had had such an attack in the previous 3 months, most of the subjects from Colombo were suffering from their first attack of malaria when investigated in the present study.

Prevalence of Multiple-clone *Plasmodium vivax* Infections

Overall, five of the 201 isolates that gave successful amplification in the PCR produced more than one amplicon each, indicating that they were MCI (see Table). For each of another 32 isolates, summation

of the lengths of the RFLP fragments resulted in a significantly greater value than the length of the corresponding undigested product, again indicating the presence of more than one *msp-3α* allele (Table). Of the isolates successfully investigated by PCR-RFLP, 19.2% of those from Anuradhapura, 20.8% of those from Kataragama-Buttala but only 13.8% of those from non-endemic Colombo appeared to come from subjects with MCI (Table; $P>0.05$).

Prevalence of Different Alleles of *msp-3α*

All but 16 of the 217 clinical isolates of *P. vivax* investigated were successfully amplified at the *msp-3α* locus. The nested PCR produced amplicons of 1200, 1500 and/or 1900 bp. The 1900-bp product ($N=146$; 72.6%) was significantly more common than the 1500-bp ($N=52$; 25.9%; $P<0.05$) or 1200-bp ($N=3$; 1.5%; $P<0.01$) products in all three study areas. As indicated previously, five isolates gave more than one amplicon, each giving amplicons of 1500 and 1900 bp (Table).

Although the undigested PCR products fell into just three sizes, the RFLP analysis yielded highly diverse size fragments and banding patterns, even for the isolates that gave only one PCR product each, indicating substantial diversity at the nucleotide level.

With all of the isolates that were successfully investigated, the largest fragments resulting from *HhaI* digestion or *AluI*

TABLE. The numbers of samples investigated and multiple-clone infections identified in each of the three study areas in Sri Lanka

Study area	No. of samples	PCR	No. of multiple-clone infections identified by:*			
			PCR-RFLP using:			
			<i>HhaI</i> only	<i>AluI</i> only	Both enzymes	Any method
Anuradhapura (endemic)	52	1	3	4	2	10
Kataragama (endemic)	91	3	8	7	1	19
Colombo (non-endemic)	58	1	1	4	2	8
All three areas	201	5	12	15	5	37

*Overall, 201 clinical isolates were successfully investigated by PCR, and 196 by PCR-RFLP.

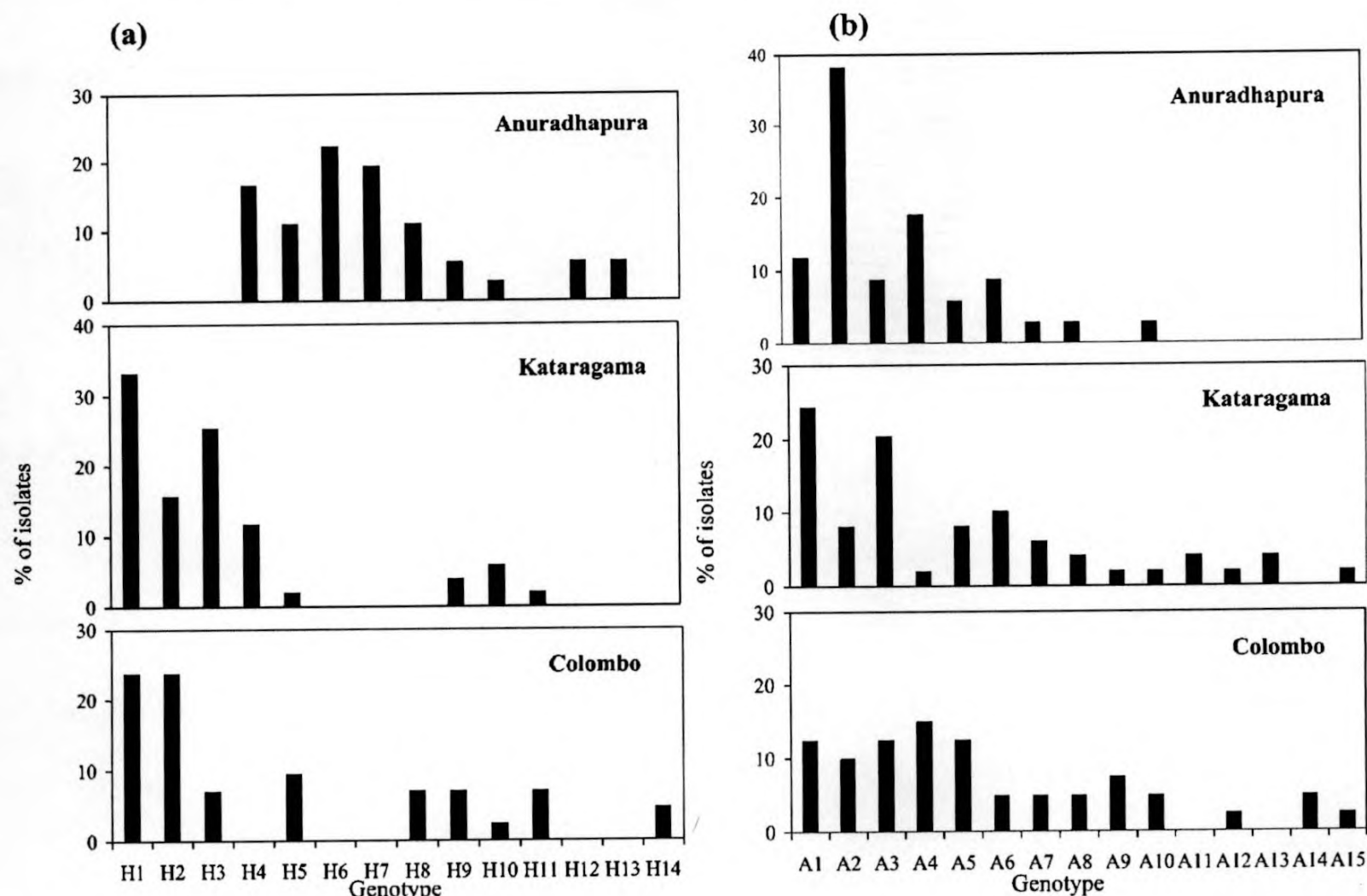


FIG. 3. Some of the results of the PCR-RFLP genotypes, showing the percentages of the single-clone isolates from each of the three sample-collection sites identified as (a) genotypes H1–H14 (after *HhaI* digestion) or (b) genotypes A1–A15 (after *AluI* digestion). Only the data for genotypes that each represented >1% of the single-clone isolates with 1900-bp alleles are shown.

digestion were of the same sizes (about 1000 and 500 bp, respectively). It was therefore the smaller fragments, of 175–600 bp with *HhaI* and 150–450 bp with *AluI*, that were used to distinguish genotypes (see below).

Genotypes Identified by PCR-RFLP

The RFLP banding patterns of 196 single-clone samples indicated 22 different genotypes with *HhaI* digestion, and 26 with *AluI*. The combined *HhaI* and *AluI* patterns resulted in 51 different genotype combinations (results not shown). The diverse genotypes were mostly confined to the 1900-bp allele. Just four genotypes from each digestion (recorded as H1–H4 for *HhaI* and A1–A4 for *AluI*) represented >50% of the infections. Genotypes H1, H2 and H3 were only detected in the samples from Kataragama–Buttala, while another three genotypes (H6, H7 and H8) were exclusively found in the samples from Anuradhapura [Fig. 3(a)]. Although these six genotypes were therefore

‘area-restricted’, they also accounted for the majority of the sampled infections in the areas where they did occur (86.3% of the single-clone isolates from Kataragama–Buttala being H1, H2 and H3 and 80.5% of such isolates from Anuradhapura being H6, H7 or H8). The A1–A4 genotypes also showed uneven distribution between the two endemic areas [Fig. 3(b)].

Genetic Diversity of *Plasmodium vivax* *msp-3α* in Sri Lanka

Direct sequencing of the 1900- ($N=8$), 1500- ($N=8$) or 1200-bp ($N=1$) amplicons, produced from 17 single-clone infections, resulted in sequences that were readable only between amino-acid positions 112 and 662 (Fig. 2) of the corresponding (*msp-3α*) sequence for the Belem strain of *P. vivax* (Rayner *et al.*, 2002). These sequences corresponded to the polymorphic alanine-rich coiled coils of domain I and the coiled coils of the heptad repeats of domain II

(Galinski *et al.*, 1999; Rayner *et al.*, 2002). The presence of the N-terminal and C-terminal variable regions in the 1900-bp allele, and the deletion of the N-terminal segment of this allele in the 1500- and 1200-bp alleles, were apparent.

While the 196 polymorphic sites identified in the 1900-bp genotype ($N=8$) gave rise to 202 mutations (Fig. 4), the 1500-bp genotype ($N=8$), with only five polymorphic sites identified, resulted in only five mutations (the limited polymorphism in the 1500-bp genotype probably resulting from the deletion within the hyper-variable region of block I). More evidence of the conserved nature of the 1500-bp allele came from the indices of nucleotide diversity (with π -values of 0.001 for this allele and 0.0555 for the 1900-bp allele) and the mean numbers of nucleotide differences (with k -values of only 1.25 for the 1500-bp allele and 88.0 for the 1900-bp).

Deletions and a large number of amino-acid substitutions were found at the *msp-3 α* locus. Interestingly, the 1900-bp genotype was represented by eight different haplotypes, resulting in high haplotype diversity ($H_d=1.000$), whereas only five haplotypes were identified in the 1500-bp allele type ($H_d=0.786$; Fig. 5).

Examination of synonymous and non-synonymous nucleotide substitutions revealed the presence of 121 non-synonymous substitutions in the 1900-bp genotype but only one such substitution (Asp/Glu) in the 1500-bp genotype. The more frequent NS substitutions within the 1900-bp genotype again indicate the presence of high variability.

Although the PCR-RFLP data for the 17 isolates investigated by sequencing indicated only 11 different haplotypes, the corresponding sequence data resulted in 13 distinct haplotypes, indicating that there was more diversity at the nucleotide level than revealed by PCR-RFLP alone.

In the phylogenetic analysis, the isolates with the 1900-bp allele were not clustered into a specific clade but were distributed throughout the tree, forming three clades

(Fig. 5). The 1500-bp isolates, in contrast, were robustly clustered into a single subgroup of a clade (Fig. 5).

Relationship between MCI and Seasonal Malaria Prevalence

During three of the four mass blood surveys carried out to evaluate the point prevalence of malarial infection, >75% of the combined populations of the three study villages were screened. The recorded prevalences and corresponding rainfall pattern are presented in Figure 6. The prevalence of *P. vivax* infections detected during the surveys ranged from 0.38% to 1.72% (Fig. 6). The corresponding range for *P. falciparum* was 0.58%–3.02%.

Comparison of the proportions of investigated clinical isolates from Kataragama-Buttala identified as multiclonal — 18 (22.5%) of the 80 isolates collected in the high-prevalence or high-transmission season (September–March) and none (0%) of the 11 isolates collected in the low-prevalence/low-transmission season (April–August) — indicated that seasonal changes in *P. vivax* prevalence may influence the occurrence of MCI.

DISCUSSION

The close association between the genetic complexity of the local malarial infections, the prevalence of MCI and transmission intensity is well established (Babiker *et al.*, 1997; Anderson *et al.*, 2000; Cristiano *et al.*, 2008). Globally, between 10.6% and 65% of the *P. vivax* infections investigated, in humans living in endemic areas, have been found to be multiclonal (Suwanabun *et al.*, 1994; Kolakovich *et al.*, 1996; Joshi *et al.*, 1997; Bruce *et al.*, 2000; Cole-Tobian *et al.*, 2002; Cui *et al.*, 2003; Kim *et al.*, 2006; Cristiano *et al.*, 2008; Moon *et al.*, 2009). Unfortunately, differences in the sampling and genotyping methods used in these investigations limit the significance of the apparent geographical variation observed.

GAATTGCAAAAAGTCAAAACGGCAAAAGATGAATCAACAACCGCAGCTAAGGATGCAGAACTGCTAAAAAGAACGCA 413
 GTGAGCGCAGGAAAGGGATTGGACGTAGCGAAGGAAGCCATAAAAAAGGCAGAAACAGCAGCAGCAGCGAAGCGAAAAAC 491
 CAAGCCGCTATAGCAGAAAAGGCAGCAGACGGCGCGAGGCAGCTGGAAAGGAAAATAAATTAGACCGATGTAAAAAGT 569
 CAAGTAAAAATTGCAGAAAGAGCGTCAACCAAAAGCTAAGGATAAAAAAACCGAAGCGGAAATAGCCGTAGAGATAGTC 647
 AAAGCAGTAGTGGCAAAGGAGGAAGCGCAAAAGGCGTCAGATGAGGCCAGAAAGGCATCCGAGAAAGCCAAGAAAGGCA 725
 CACGCGAAAGCGCAAAAGGCTTCTGAT.....ACAACAAA 803
 ACGGTAGAAACGTTCAAAACAAACGCAGAAAGCTGCGGCAAAAATGCAAAAGGAAAGGGCGGAAATGCCAACGAAGCG 881
 GCAACGGATGCAGAAAGGTGCTAACGAATTAAGCGCAGCAAAACAAAAGCGAAGACGCAGAAAGCGGCCGCTAAAGAA 959
 GCGAAAAAGGAACAGGTAAAAGCAGAAATAGCTGCCGAAGTGGCGAAGCCAAAAGTTGCAAAAGAAGAAGCAGAAAGAA 1037
 GCACAAAAAAGCAGAAAGAGGCAAAAGAAATCGTAGACAAAATAGCAAAGATTCTGAAGTACCAGAGGCCCAAAAG 1115
 GCAGCAGAAATTGCGCACTGAAACGGTTAAAAGGCAACCACCGCGGCGACAGAAAGCAGGCAAAAATGCGCAAGAAGCT 1193
 GAAAGTCAACCAAGAAAGCAGAAAGCAAGCAGCAACGTCAGACGCGGTAAAAGGAAAAGCCGATGCTGCAGAAAAA 1271
 GCCGCAGGAGAAGCAAAAAAGCATCGATCGAAACGGAAATAGCAATTGAAGTAGCAAAAGGCAGAAAGTGCTGAACGCA 1349
 GAAGTAAAAAGACAGCCCAAGAAAGCGAAAAAGACGCCACGGAGGCTAAGGAGCAGGCAGAAAAGGCTAAGGCAGCA 1427
 GCGGAAGAGGCAAAAGACGCACGGAGAAGAGCAGAAAAGGTAGGAGAGTCAACAAAAGCACATTTCAGATGAAGCACAG 1505
 CAAGAAAATAAAAAAGCAAAAGGACGCCTCGGAAGAGCAGAAAAAGAGAGCGGTGGACGCATTGGAAGAAGCGTATGCA 1583
 GTGGAAGCACATCTGGCAAGAACCAAAATGCAGCAGAGTCAGCCAAAAGTGCAACAGATATGAGTGAATTAGAAAAG 1661
 GCAAAAGAGGAAGCGATAGATGCAGCAAAATATGCCCATCAAAAGTGGTTAAAAGCAACCCAGGCAGCTACCATTGCA 1739
 AAAGAAAAGAAGGAAGCTGCAAAAGTAGCCGCTGAGAAAGGCACAAACTGCAGCAAACGTTGTAAAAGATAAGGCAGCC 1817
 AAGGCTGAAGCGAAGAAAGGCAGAAACAGAAAGCGGTAAAGGCAGCGGTAGAAGCGAGAGCGGCGGCGGAGGAAGCGAAG 1895
 CAAGAGGCAGCAAAAGTAGGGGCATCGAAAGAACCACAAGAACTAAAAATAAAGCAAAATGTGGAAGCAGAAAGCAACA 1973
 GGAAATGAAGCAAAAAAGGCAGAAAGATGCCGCGGAGGAAGGTAAAGAAAGCAGCCAAAAAAGCAAAATGAAGCA

FIG. 4. Nucleotide polymorphisms in blocks I and II of the *msp-3α* genes of 17 Sri Lankan isolates. The consensus sequence was obtained from aligning the nucleotide sequences of the 17 *msp-3α* sequences, the polymorphic sites being highlighted here by grey shading.



FIG. 5. Phylogenetic tree based on the *msp-3α* genes of 17 Sri Lankan isolates of *Plasmodium vivax*. The tree was constructed using the neighbour-joining method. The *msp-3α* alleles investigated were represented by 1900-, 1500- or 1200-bp amplicons in the PCR. Each isolate number on the phylogenetic tree is the relevant GenBank accession number.

The present study appears to be the first large-scale investigation on the genetic complexity of *P. vivax* infections in Sri Lanka. The proportion of infections found to be multiclonal (20%) and the size polymorphism and diverse genotypes identified in studies at the *msp-3α* locus indicate a high degree of genetic complexity in the local *P. vivax* infections, on an island with generally low and unstable malarial transmission (Rajendram and Jayewickreme, 1951; Mendis *et al.*, 1990).

In earlier studies based on serology (Udagama *et al.*, 1990) or sequence analysis (Premawansa *et al.*, 1993), only about 10% of *P. vivax* infections in Sri Lankans were

identified as multiclonal. As the serological screening (Udagama *et al.*, 1990) was mostly performed on isolates that differed from those used to produce the monoclonal antibodies employed, certain genotypes may not have been detected. Surprisingly, in a recently published genotyping study using microsatellites, the proportion of the investigated *P. vivax* isolates identified as MCI varied from 9.1% in 2003 to 60.0% in 2005, although the sample sizes (22 and 20, respectively) were fairly small (Karunaweera *et al.*, 2008).

In the present study, the proportion of investigated isolates found to be multiclonal (20%) was higher than the values recorded

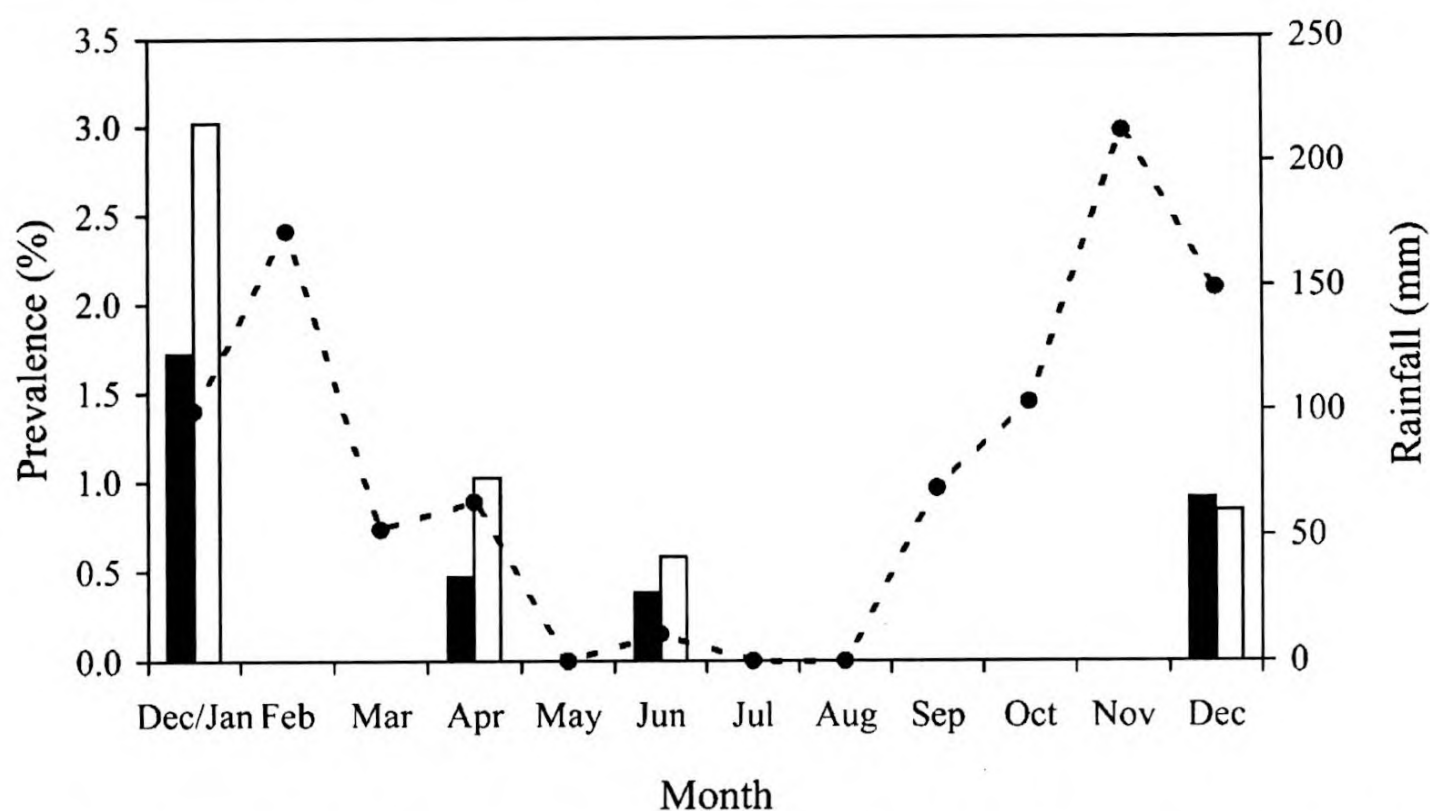


FIG. 6. The point prevalences of *Plasmodium vivax* (■) and *P. falciparum* (□) infection in humans living in the malaria-endemic Kataragama–Buttala area, as measured in four mass blood surveys (in December 1998–January 1999, April 1999, June 1999 and December 1999), and the corresponding rainfall (●) in the same area.

in Sri Lanka prior to 2005. As this value is based on a relatively large sample and the genotyping of the *msp-3α* locus (which is generally considered to be a reliable molecular epidemiological marker; Kim *et al.*, 2006; Zakeri *et al.*, 2006; Cristiano *et al.*, 2008; Véron *et al.*, 2009) by PCR–RFLP, it may be the best representation of the local situation yet produced. The 20% frequency recorded in the present study is similar to the value recorded in other regions with hypo-endemic *P. vivax* malaria, including Thailand and Venezuela (Bruce *et al.*, 1999; Cui *et al.*, 2003; Ord *et al.*, 2005). The number of MCI detected in the present study may, however, under-estimate the number represented by the investigated samples, since low parasitaemias may hinder the PCR-based amplification of a subpatent secondary strain (De Roode *et al.*, 2005; Havryliuk *et al.*, 2008).

The distribution of human malaria across Sri Lanka is mainly determined by climatic factors that are essential to the development of the vector mosquitoes, with the island's dry zone traditionally being endemic. Those Sri Lankans who live in non-endemic areas, such as Colombo, only become infected with *P. vivax* when they visit endemic areas.

Three basic factors may be responsible for a multiple-clone *Plasmodium* infection in a human (Cui *et al.*, 2003): the super-infection of the human host, the simultaneous co-infection of the human host, or the somatic mutation of a single clone of parasites in the human host. When Sri Lankans are infected in endemic areas, the local residents and visitors from non-endemic areas are, presumably, equally likely to be infected by the bite of a mosquito carrying a multiple-clone infection. Although the risk of super-infection may clearly be greater among the infected residents of endemic areas than among the infected residents of non-endemic areas, the general level of transmission in Sri Lanka is so low that super-infection must be a rare event even in the endemic areas. In Kataragama, for example, the chance of an individual being bitten by an infectious mosquito on a single night has been estimated to be only one in 500 (Mendis *et al.*, 1990). This may explain why, in the present study, among the human *P. vivax* infections investigated in each area, MCI were only slightly (and not significantly) less common in non-endemic Colombo than in Anuradhapura or Kataragama–Buttala. In

an earlier study in Sri Lanka, MCI were also found to be rarer among isolates from non-endemic Colombo than among those from endemic settings (Premawansa *et al.*, 1993). The vast majority of MCI that are detected in non-endemic areas, in people who have, in general, only paid brief visits to areas with malarial transmission, are presumably the results of bites by mosquitoes that have MCI themselves (Druilhe *et al.*, 1998).

Few residents of Kataragama (or, presumably, other endemic villages in Sri Lanka) have acquired clinical immunity to *P. vivax* infection and few seek prompt treatment for such infection (Gunawardena *et al.*, 1994). In Sri Lanka, relapses may occur 8–24 weeks after primary infections with *P. vivax* (Fonseka and Mendis, 1987), and a general reluctance to complete the recommended primaquine regimen presumably allows relapse infections to develop (Havryliuk and Ferreira, 2009). Thus, multiclonal infections may result when relapse infections develop at the same time as single-clone re-infections.

Although Fonseka and Mendis (1987) attributed 18% of clinical *P. vivax* infections from Colombo to relapses, four (50%) of the MCI from the city identified in the present study were first infections and the rest were probably the result of re-infection (as all four cases had recently visited malarious areas). The contribution of relapse infections to MCI in this urban setting therefore seems to be slight.

The geographical variation seen, in the present study, in the distributions of some of the *P. vivax* genotypes (probably resulting from genetic drift) has not been reported previously in Sri Lanka. Given the generally low transmission intensity, it is not surprising that some genotypes common in Anuradhapura were rare or undetected 250 km away, in Kataragama–Buttala, and vice versa. With little transmission occurring and only 20% of *P. vivax* infections in the local human populations being multiclonal, unrelated *P. vivax* parasites rarely co-exist in the same mosquito bloodmeal. The

resultant parasite inbreeding, which occurs in other areas with low levels of transmission, such as South America (Anderson *et al.*, 2000), helps to restrict the geographical spread of particular genotypes. In contrast, parasite outbreeding is more usual in areas with high levels of transmission, such as much of Africa, and tends to reduce the geographical variation in the predominant genotypes (Anderson *et al.*, 2000).

Knowledge of the genotypes that predominate in a particular area may allow the geographical origin of a human infection to be identified. In the present study, for example, it was sometimes possible to identify which of the Colombo subjects had probably been infected in the Kataragama–Buttala area and which in the Anuradhapura area, from the *P. vivax* genotypes that these subjects harboured. In each case, the identified ‘probable source of infection’ matched the travel history of the subject involved (data not shown).

The results of the four mass blood surveys that were carried out in the Kataragama–Buttala area provide a longitudinal view of the prevalence of malarial infection throughout a year. The apparent absence of MCI during the months of June and July, when rainfall and *P. vivax* point prevalence were both at their seasonal minima, indicates that there might be a significant association between malaria transmission intensity and the occurrence of MCI, at least in the Kataragama–Buttala area.

When pair-wise indices of nucleotide diversity (π) were obtained using various *P. vivax* merozoite proteins but the same clinical isolates as investigated in the current study (P. V. Udagama-Randeniya, unpubl. obs.), the value for *msp-3 α* ($\pi=0.0590$) was found to be higher than that for *dbpII* (0.0098), *ama-1* (0.0095) or *msp-1p₄₂* (0.0231). Relatively high levels of diversity in *msp-3 α* were also reported by Mascorro *et al.* (2005). The overall haplotype diversity (Hd) recorded, in the present study, for the 1900-bp fragment indicates very high levels of genetic diversity, at the *msp-3 α* locus,

within Sri Lankan *P. vivax*. Together, the high values recorded, in the present study, for pair-wise nucleotide diversity, haplotype diversity (for the 1900-bp amplicon) and prevalence of the 1900-bp allele provide further evidence for the suitability of the *msp-3α* gene as a genetic marker in the evaluation of the genetic complexity of *P. vivax* infections, at least in Sri Lanka.

The observation that, in the phylogenetic analysis (Fig. 5), the 1900-bp allele formed three clades that were distributed throughout the tree whereas the 1500-bp allele was restricted to a single subgroup of one clade may indicate that the 1900-bp version is the ancestral form and has had far longer to diverge (the 1500- and 1200-bp being derived forms that evolved more recently).

In conclusion, the present results indicate that about 20% of the *P. vivax* infections in the humans in two malaria-endemic areas of Sri Lanka (with low and unstable malaria transmission) are multiclonal. Super infections (presumably more common in endemic areas than in non-endemic) and relapses contribute to multiple-clone infections. The prevailing genetic diversity, the frequency of MCI and the association of particular genotypes with particular endemic areas highlight the complexity of the *P. vivax* infections in Sri Lanka.

ACKNOWLEDGEMENTS. This research received financial support from the Sri Lankan National Science Foundation (via grant NSF/RG/2005/HS/06), the Sri Lankan National Research Council (via grant NRC-05-34) and the UNDP/World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases (as project ID 980270). The authors are grateful to all those who donated blood. The medical superintendents, physicians, housemen and nursing staff of the General Hospital at Anuradhapura and the National Hospital Sri Lanka are thanked for their co-operation. The assistance rendered by L. Perera, S. Weerasignhe and J. Rajakaruna

(all of the Malaria Research Unit at the University of Colombo's Department of Parasitology), and by staff members of the University of Colombo's Department of Zoology was deeply appreciated. The authors are particularly grateful to the Director of the University of Colombo's Institute of Biochemistry Molecular Biology and Biotechnology.

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Genetic complexity of *Plasmodium vivax* infections in Sri Lanka

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Using *Plasmodium vivax* Merozoite Surface Protein-3 α (PvMSP-3 α) marker, the existing genetic complexity of *P. vivax* infections was evaluated in individuals (N=146) from two malaria endemic areas, Kataragama (N=68), Anuradhapura (N=39) and from Colombo (N=39), a non-endemic region of Sri Lanka. A combination of polymerase chain reaction / restriction fragment length polymorphism (PCR/RFLP) techniques was used for this assessment.

Undigested PCR products showed a major size polymorphism. The product sizes were approximately 1900, 1500 and 1200 bp among which the former was the most predominant (72.8%), while the other two accounted for 25.8% and 1.4%, respectively. The predominance of the 1900 bp product was more significant [Chi square test: (with 1500 bp; P<0.05) and (with 1200 bp; P<0.01)] than the other two products in all three study areas. Despite the presence of only three size variations in undigested PCR products, RFLP analysis yielded highly diverse fragment sizes and banding patterns for all samples. The RFLP patterns of all isolates showed size conservation of the largest fragment obtained from *Hha* I digestion (~1,000 bp) and *Afu* I digestion (~500 bp), while smaller fragments showed considerable size variation. The summation of RFLP fragments of 27 samples (19%), was significantly greater than the size of the undigested product, indicating the presence of more than one PvMSP-3 α allele in these infections. The number of these multiclonal infections was significantly higher (Chi square test; P<0.05) in the two endemic areas as compared to the non-endemic area. From the samples in which single clonal infections were detected, where smaller fragments resulted from RFLP digestion, at least 10 different banding patterns from each restriction enzyme were detected, indicating the presence of a substantially high diversity at the nucleotide level.

These results demonstrate the prevailing genetic complexity among individual *P. vivax* infections in Sri Lanka, and also confirm the suitability of utilizing PvMSP-3 α locus to analyze the degree of polymorphism as well as clonality of *P. vivax* infections in this geographical region.

Financial support by the National Science Foundation, Sri Lanka [Grant NSF/RG/2005/HS/06 and scholarship for PHP (NSF/SCH/2004/07)] is acknowledged.

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B3 - IgM antibody response to *Plasmodium vivax* Duffy Binding Protein in Sri Lanka

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Recombinant protein PvRII, representing native *Plasmodium vivax* Duffy binding Protein, a potential vaccine candidate, was used in an antibody sandwich ELISA for the quantification of the IgM response in patients with acute vivax malaria. The test groups were from two malaria endemic areas Anuradhapura (n=33) and Kataragama (n=39), and from non-endemic area Colombo (n=54).

No significant difference was detected in IgM prevalence (70%, 74% and 80% for Anuradhapura, Kataragama and Colombo, respectively) of residents among all three-test areas (Chi square test, $P > 0.05$). This was also reflected for anti-PvRII IgM magnitudes (Mann-Whitney U test, $P > 0.05$). In Colombo, the anti-PvRII IgM prevalence (Chi-square, $P > 0.05$) but not the magnitude (Mann-Whitney U test, $P > 0.05$) of previously exposed (PE) individuals (n=30) was significantly higher than of the previously not exposed (PNE) individuals (n=12). Anti-PvRII IgM magnitude in all test populations showed no significant association with age of the individual, their parasite density, duration between present and penultimate malaria infection, and the number of past malaria infections suffered. On examining these data with the

B2 - Specificity of the antibody response to conformational and linear epitopes of *Plasmodium vivax* Duffy Binding Protein in Sri Lanka.

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Erythrocyte invasion by *Plasmodium vivax* is completely dependant on binding to the Duffy blood group antigen by the parasite Duffy Binding Protein (DBP). Thus, DBP is identified as a prime vaccine candidate against vivax malaria. *E. coli* expressed recombinant protein PvR11, representing the critical binding region of native *P. vivax* DBP, was used in a reduction sensitive ELISA to examine the natural antibody response to conformational and linear epitopes of DBP. The serum samples collected from patients microscopically confirmed for acute vivax malaria that previously tested positive for total (IgG + IgM) anti-PvR11 antibodies, represented two malaria endemic areas, Anuradhapura (N=33) and Kataragama (N=39), and non-endemic Colombo (N=40).

Although a conformation sensitive anti-PvR11 monoclonal antibody 2H10 reacted with the non-reduced PvR11 but not with its reduced form, the test sera did partially react with the reduced PvR11. The OD values obtained with the reduced PvR11 elicited a suppression of reactivity compared to the non-reduced antigen by 53%, 47% and 46% from Colombo, Anuradhapura and Kataragama, respectively. This suppression was not significantly different (Chi Square test, $P > 0.5$) among the test areas. The partial reactivity of the serum samples with the reduced antigen indicated the recognition of linear B cell epitopes of PvR11. The antibody response to the linear epitopes of previously unexposed individuals from Colombo (N=12) was

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K76T point mutation of chloroquine resistance transporter gene: Is it a potential molecular marker for chloroquine resistance in Sri Lankan *Plasmodium falciparum* isolates?H A C Hapuarachchi^{1*}, M Y D Dayanath², S Abeysundara¹, A Manamperi², W Abeyewickreme¹ and N R de Silva¹¹Department of Parasitology, Faculty of Medicine, University of Kelaniya, P.O. Box 6, Thalagolla Road, Ragama.²Molecular Medicine Unit, Faculty of Medicine, University of Kelaniya, P.O.Box 6, Thalagolla Road, Ragama.

Current evidence suggests that K76T mutation of chloroquine resistance transporter (*Pfcr*t) gene may be used as a molecular marker for chloroquine (CQ) resistance of *P. falciparum*. This study was carried out to determine the frequency of K76T mutation of *Pfcr*t gene in Sri Lankan *P. falciparum* isolates collected from Mannar district in the Northern Province. Mutation patterns were compared with *in vitro* and *in vivo* CQ failure rates for this parasite population to analyze its association with resistance to CQ and to calculate the genotype-resistance (GRI) and genotype-failure (GFI) indices for K76T mutation. *P. falciparum* DNA was extracted from dried blood spots using a QiaAmp DNA Blood Mini Kit. Mutation patterns at 76 codon of *Pfcr*t of field isolates were detected using a polymerase chain reaction – restriction fragment length polymorphism assay. Parasite isolates were categorized into wild (sensitive) and mutant (resistant) types based on the banding patterns of digested products on 2% agarose gels. GRI and GFI indices were calculated for this parasite population. Of 38 CQ resistant isolates, 86.8% (N = 33) showed the mutant allele (K76T) at codon 76 of *Pfcr*t. There was a statistically significant association between the presence of K76T mutation and *in vivo* resistance to CQ ($\chi^2 = 5.11$, $p = 0.02$). Of 33 sensitive isolates, 39.4% (N = 20) possessed the wild type allele at the same codon position. The calculated GRI and GFI indices were 1.13 and 1.38 respectively for this area. Even though results show a statistically significant association between the presence of K76T allele and CQ treatment failure of *P. falciparum* isolates in the study population, its mere presence alone does not seem to correlate with resistance to CQ. Therefore, mutations at other codons of *Pfcr*t shown to accompany K76T mutation as well as those in *P. falciparum* multi drug resistance 1 (*Pfmdr*1) gene need to be analyzed to determine whether other mutations play a role together with K76T in clinically resistant Sri Lankan parasite isolates. More studies are required to validate the calculated GRI and GFI values, which may be used to predict the therapeutic failure of CQ in a particular area in Sri Lanka in the future.

Acknowledgement: National Science Foundation Research grant SIDA/2005/BT/03 and by the IAEA TC Project SRL 06/028

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Patterns of anti-PvRII IgG and IgG isotype responses to *Plasmodium vivax* Duffy Binding Protein in Sri LankaPrasad H Premaratne¹, K V G Sajani Dias¹, Shiroma M Handunnetti², Shams Yazdani³, Chetan Chitins³, Preethi V Udagama-Randeniya^{1*}¹Department of Zoology, Faculty of Science, University of Colombo, Sri Lanka.²Malaria Research Unit, Faculty of Medicine, University of Colombo, Sri Lanka.³International Center for Genetic Engineering and Biotechnology, New Delhi, India.

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Exploring the nature of the antibody response to potential vaccine candidates, during natural malaria infections would provide essential information for vaccination strategies. Recombinant protein PvRII, that represents native *Plasmodium vivax* Duffy Binding Protein, was used in an ELISA to assess the magnitudes of IgG and IgG isotype responses in acute vivax malaria patients. Serum samples that screened positive for anti-PvRII total (IgG+IgM) antibodies in a previous study were pre-selected for this assay from non-endemic Colombo (N=58), and from two endemic areas, Anuradhapura and Kataragama (N=48 from each area). The percentage of responders (prevalence) with anti-PvRII IgG was 71%, 79% and 67% from Colombo, Anuradhapura and Kataragama, respectively. No significant differences were detected among the proportion of responders (Chi square, $P>0.05$) and mean anti-PvRII IgG magnitudes (Kruskal-Wallis, $P>0.05$) among the three study populations. Cytophilic IgG3 was the most prevalent IgG isotype in all three-study cohorts. No significant difference was observed for prevalence of IgG isotypes between the three areas, with the exception of the significantly higher prevalence of IgG3 in Colombo than in Kataragama (Chi square, $P<0.05$). The prevalence of IgG3 was significantly higher than the other three isotypes within the residents of Colombo. However, in both endemic areas no significant difference was apparent between the prevalence of IgG3 and IgG1 (McNemar, $P<0.05$). In all test areas, prevalence of restricted IgG3 responses to PvRII was significantly higher (Chi square, $P<0.05$) than for responses restricted to each of the other 03 isotypes. Significantly higher magnitudes of IgG1 than the other IgG isotypes (Wilcoxon Signed ranks, $P<0.05$) were detected within each test area. A significantly higher IgG1 magnitude was detected in Colombo than in Kataragama (Mann-Whitney U Test, $P<0.05$). Both the prevalence and magnitudes of total IgG and of IgG isotypes were independent of age of patients or their previous exposure to malaria or to their parasite burden. These results may imply that cytophilic antibody isotypes IgG1 and IgG3 may play important roles with regard to the immune response to PvDBP in Sri Lanka.

Acknowledgement: National Science Foundation of Sri Lanka (grant# NSF/RG/HS/06 and scholarship for PHP [NSF/Sch/2004/07])

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Nocturnal human biting behavior of *Anopheles culicifacies* in a hydro electric power project area situated in a sylvatic environment in the Central Province of Sri Lanka

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Nocturnal human biting behavior of *Anopheles culicifacies* was studied from January 2006 - March 2007 at the Victoria hydro electric power generation project area in the Central Province of Sri Lanka. Monthly collections of indoor and outdoor, landing and biting, mosquitoes were made from 1700 - 0600 hours (13 hours) using 3 - 4 indoor and 4 -8 outdoor human baits. In each collection, human baits were seated with exposed hands and legs below the knees. Hourly collections of landing and biting mosquitoes on the exposed surfaces of the body using torches and aspirators were made and mosquitoes were identified to species. Data was summarised as the number of *An. culicifacies* collected indoors and outdoors each hour/bait separately. The majority (97.83%) of *An. culicifacies* bit outdoors as compared to indoors. There were monthly variations in the biting habits with the highest

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Molecular Ecology of *vivax* malaria: genetic diversity of a malaria vaccine candidate antigen, Apical Membrane Antigen -1, in Sri Lanka.

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Of the four human malaria parasites, *Plasmodium vivax* is usually considered a benign parasite that threatens almost 40% of the world's population. It accounts for 70 to 80 million malaria infections each year in Asia, Oceania, and South America, but is rare in most of Africa. The greatest burden was observed in Southeast Asia (52%), Eastern Mediterranean (15%), and South America (13%). The predominant human malaria species in Sri Lanka is *P. vivax*.

Control of malaria is hampered due to resistance of parasites to anti malarial drugs and resistance of mosquito vectors to insecticides. Therefore, a viable option is to develop an effective vaccine. Diversity studies can be made to provide great opportunities to test parasite-specific questions. One of the strongest influences of Molecular Ecology in the field of Parasitology has been on the study of parasite population genetics. Vaccine development for malaria will be complicated due to many reasons, among which the extensive antigenic diversity (genetic diversity) of a potential malaria vaccine candidate in different parasite 'strains' will pose a critical problem.

Symptoms of malaria are due to the life cycle stages of the parasite in the human red blood cells (RBC). The extra cellular merozoite stage of *Plasmodium* is an important vaccine target. The RBC cycle of the malaria parasite is continued when the merozoite enters an uninfected RBC. Certain parasite proteins are important for the successful invasion of the merozoite into the RBC. One such protein, which is an important vaccine candidate, is the *P. vivax* Apical Membrane Antigen-1.

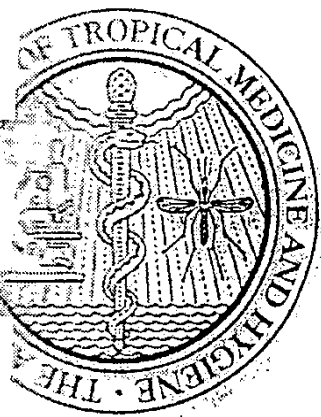
We studied the genetic diversity of Domain II of *P. vivax* Apical Membrane Antigen-1 (PvAMA-1), in different parasite isolates, where a single isolate represents the parasite population of an infected individual. Forty four single clonal *P. vivax* isolates, 31 from two malaria endemic areas (EA) Anuradhapura and Kataragama, and 13 from a non-endemic area (NEA), Colombo, were amplified and sequenced. These sequences were further analyzed with other available geographically different isolates of Domain II PvAMA1 sequences (Sal-I, 11 Indian isolates, one each from the Philippines [PH84] and from Brisbane [PVQ]), to examine population diversity both within and between geographical areas.

Thirteen distinct haplotypes with respect to this antigen were identified from the Sri Lankan isolates, which are reported for the first time. Comparison of the 19 haplotypes observed in the Sri Lankan isolates revealed, 7 isolates similar to Sal-I, and one similar to an Indian isolate. Six haplotypes have been previously reported by a study done by our group.

Analysis of 44 Sri Lankan isolates showed 16 polymorphic sites and 17 point mutations. 9 new non-synonymous replacements were distinctive to the Sri Lankan isolates only. Even though not significant, diversity was comparatively higher in the NEA to that of EA's. To determine whether natural selection contributes to the diversity in these three areas, the ratio of nonsynonymous to synonymous substitutions (dN/dS) was evaluated. It revealed that, the protein is most subjected to diversifying selection in the two Endemic areas.

Therefore, understanding the genetics of a parasite population can assist greatly in the development of vaccines which are much more effective and long lasting.

Financial support by NSF/RG/2005/HS/06 and NRC Grant 05-34 are acknowledged.



THE AMERICAN JOURNAL OF
***Tropical Medicine
and Hygiene***

Official Journal of the American Society of Tropical Medicine and Hygiene

Volume 77

November 2007

Number 5

Abstract Book

American Society of Tropical Medicine and Hygiene

56th Annual Meeting



November 4–8, 2007

Philadelphia Marriott Downtown

Philadelphia, Pennsylvania USA

Supplement to

The American Journal of
Tropical Medicine and Hygiene

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SPECIFIC *PLASMODIUM* ELIMINATION DURING A SECOND INFECTION IN CBA/CA MICE

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In malaria endemic areas individuals are susceptible to several infections before they develop a protective immunity, the basis of this susceptibility and the precise nature of the immune mechanisms that control the parasitaemia during the first and subsequent infections are still not well understood, however, they are relevant to understand the immunity to malaria. In this work we studied how a first infection with *Plasmodium yoelii* 17XL affect the dynamic of the parasitaemia during a second infection with the homologous parasite, or with *P. chabaudi* AS or with the mixture of *P. chabaudi* AS and *P. yoelii* 17XL in CBA/CA mice using a nested PCR. A batch of CBA/CA mice was infected with 5×10^4 parasitized erythrocytes with *Plasmodium yoelii* 17XL. On day 7 post-infection, mice were treated with a therapeutic dose of pyrimethamine, 8 weeks later, mice were split into three groups, the first was re-infected with 5×10^4 parasitized erythrocytes of the homologous parasite, the second group was re-infected with *P. chabaudi* AS and the third with the mixture of both parasites. Parasitaemia was measured in Giemsa stain blood films and by using a specific nested PCR. We also studied some of the pathology parameters as weight lost, splenic index and haemoglobin concentration in the re-infected mice. The results show that there was a specific parasite elimination since mice reinfected with the homologous *Plasmodium* developed lower parasitaemia levels than mice infected with the heterologous parasite. Interestingly mice reinfected with the mixture of both parasites cleared the parasitaemia almost at the same time than mice infected with the homologous *Plasmodium*. In spite of the ability to clear the parasitaemia developed by mice infected with the homologous parasite, this group of mice showed almost the same levels of haemoglobin concentration and weight lost that mice infected with the heterologous parasite. The results of splenic index showed an increment in all groups of mice, at the beginning of re-infection it was higher in mice re-infected with *P. yoelii* 17XL but at the end of re-infection the splenic index was higher in mice infected with *P. chabaudi* AS. In this work we showed that during a re-infection even when protection against the homologous parasite has been developed still remains strong spleen activation probably due to exposition to different or new *Plasmodium* antigens, which explains at least in part the need of several infections to get a solid immunity.

(CMCIP Abstract)

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ROLE OF COMPLEMENT AND COMPLEMENT REGULATORY PROTEINS IN SEVERE ANEMIA CAUSED BY *PLASMODIUM CHABAUDI*

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Malaria accounts for about 1-2 million deaths per year, with the majority due to complicated *Plasmodium falciparum* malaria, such as severe malaria-associated anemia. Not much is understood about the pathogenesis of this anemia. C57BL/6 mice inoculated with 10^6 *P. chabaudi* AS-infected red blood cells experience a primary peak of parasitemia and severe anemia at approximately 6-7 days post-infection. After one week of recovery, a secondary peak with much lower parasitemia occurs with an additional hematocrit drop. We will present data on the role of complement (C3), red cell complement regulatory proteins and anti-malarial antibody levels in the development of anemia

in this model by comparing the infection in wild-type animals and complement (C3) and complement regulatory protein knockouts.

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FC γ RECEPTOR POLYMORPHISMS IN GHANAIAN CHILDREN WITH CLINICAL MALARIA

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Multiplication of the blood-stage of the malaria parasite and host factors are responsible for the pathogenesis of malaria. Host factors include immune factors and genetic factors such as polymorphisms in Fc γ receptors. Acquired immunity to malaria is dependent on age and exposure. Cytophilic antibodies (IgG1 and IgG3) play a major role in effecting acquired immunity however, in some studies IgG2 have been found to be associated with protection against clinical malaria because of its high affinity for certain polymorphisms of Fc γ RIIA and Fc γ RIIB. Fc γ receptors, which are found on myeloid cells such as mononuclear phagocytes and neutrophils, bind to the Fc portion of immunoglobulin (Ig), leading to phagocytosis and parasite killing thus mediating immune responses. The gene that codes for this receptor is polymorphic and is associated with predisposition to malaria infection. In this study we investigated the roles of the Fc γ RIIA and Fc γ RIIB polymorphism in the pathogenesis of clinical malaria in Ghanaian children. The study population consisted of a cohort of 210 children aged, three to ten years from Dodowa in Ghana, who were followed up in longitudinal cross sectional study under the Afro-immunoassay morbidity surveillance for eight months. PCR-RFLP was used to characterize these polymorphisms in our study population. The data was related to the clinical data from the morbidity surveillance, and also to immune responses against some vaccine candidate blood-stage antigens. We present the results and discussion of the study.

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IMMUNOEPIDEMIOLOGY OF PVR11, A PUTATIVE VACCINE CANDIDATE REPRESENTING *PLASMODIUM VIVAX* DUFFY BINDING PROTEIN, IN SRI LANKA

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Study of naturally acquired antibody responses to a potential vaccine candidate is imperative to provide insights to vaccine development. Recombinant protein Pvr11, a putative vaccine candidate representing region II of native *Plasmodium vivax* Duffy Binding Protein (DBP), was used in ELISA to examine the total (IgG+IgM), IgM and IgG isotype antibody responses. A reduction sensitive ELISA was performed for total antibodies. Sera were collected from acute vivax malaria patients from two endemic areas (EAs) where low and unstable malaria conditions prevail, and from a non-endemic area (NEA) in Sri Lanka. Prevalence of total antibodies was 60% from Colombo (NEA; N=111), 46% from Anuradhapura (EA; N=94) and 41% from Kataragama (EA; N=106). Significantly higher prevalence (Chi square, $P < 0.05$) and magnitude (ANOVA, $P < 0.01$) of total antibodies were recorded from NEA compared with EAs. Total antibody parameters in all test populations were independent of age of individuals, parasite density and previous exposure. Conformation sensitive anti-Pvr11 monoclonal antibody 2H10 reacted only with non-reduced Pvr11 but not with its reduced form. Test sera partially reacted with reduced Pvr11, and this indicating the recognition of linear B cell epitopes. A parallel increase in total antibody response to Pvr11 linear epitopes with increasing exposure was detected in residents of Kataragama. No significant difference was detected among test populations either in anti-Pvr11 IgM prevalence (Chi square, $P > 0.05$) or magnitude (ANOVA, $P > 0.05$). Prevalence of IgG3 (Chi

($p < 0.05$) and magnitude of IgG1 (Wilcoxon Signed Rank test, $p < 0.05$) were significantly higher compared with other IgG isotypes in both areas. An increase in prevalence of IgG isotypes in parallel with increasing exposure was recorded from Kataragama. An antibody shift from IgG1 to IgG2 and IgG3 to a cytophilic IgG3 with increasing exposure detected in both endemic areas. MSP-1₁₉ and PvAMA-1 using the same battery of serum, was not affected by PvRII.

(ACMCI Abstract)

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INTERFERON γ RESPONSE TO A T-CELL EPIOTOPE OF *PLASMODIUM FALCIPARUM* GLUTAMATE RICH PROTEIN CORRELATES WITH PROTECTION FROM CLINICAL MALARIA IN GHANAIA CHILDREN

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Glutamate Rich Protein (GLURP) is a potential blood stage malaria vaccine antigen. T-cell response against this antigen is important for initiation and maintenance of long-lasting antibody response. In this study, we investigated responses to peptides from the conserved region of GLURP in relation to protection from clinical malaria. Randomly selected blood samples from a cohort of Ghanaian children aged 1-10 years were used. They were actively and passively followed for a malaria season and categorized as having at least one episode of malaria (susceptible), and those who did not have any episode of malaria (non-susceptible). Cryo-preserved Peripheral Blood Mononuclear Cells (PBMC) of non-susceptible children were stimulated in a 6-day culture with 4 overlapping peptides (LR129, LR130, MR186, MR187) and cytokines (IL-10 and IFN γ). IFN γ responses in culture supernatants. PBMC's from 14 non-exposed children were used as controls. IFN γ responses to GLURP LR129 and LR130 peptides in the Ghanaian children were significantly higher than their levels in Danish controls. Of the 4 GLURP peptides tested, only LR129 was assessed in culture supernatant, IFN γ responses to MR186 were significantly higher (p=0.003), suggesting the importance of this epitope in the induction of protective memory responses. This epitope of GLURP should therefore be included in a future GLURP specific vaccine.

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ANTIBODY RESPONSES TO MODIFIED *PLASMODIUM FALCIPARUM* MSP1₁₉ ANTIGENS IN PEOPLE PREVIOUSLY EXPOSED TO NATURAL MALARIA INFECTION

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The C-terminal region of the merozoite surface protein (MSP-1₁₉) is recognized by processing-inhibitory and blocking monoclonal antibodies. We have previously modified to yield proteins that can bind

MSP1 processing inhibitory antibodies but not blocking antibodies. It was unclear whether these modifications would affect critical T cell epitopes on MSP1₁₉, therefore, *in-vitro* assays for T cell responses to wildtype MSP1₁₉ and a panel of site-directed mutagenetically modified antigens was studied in individuals naturally exposed to *Plasmodium falciparum* infection. All the mutants tested stimulated T cells from exposed individuals but not in malaria naïve individuals. Interestingly, stimulation indices for the T cell responses induced by some of the mutants were at least twofold higher than those elicited by the wildtype MSP1-protein. A mutant protein with four-amino-acid-substitution: Glu270Tyr, Leu310Arg, Tyr340Ser and Glu430Leu (27+31+34+43; residues numbered from the N-terminus of MSP1₁₉) had the highest T cell stimulation index (SI=360) with prevalence of 57.1% responders. This study suggests that this specific mutant MSP1₁₉ 27+31+34+43, which also abrogate the binding of all known blocking monoclonal antibodies without affecting the processing-inhibitory antibody epitope, might be a better choice for an MSP1-based malaria vaccine.

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ANTIBODY RESPONSES TO THE MSP-1 COMPLEX PROTEINS IN CEREBRAL MALARIA PATIENTS IN INDIA

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Humoral immune responses to the merozoite surface protein-1 (MSP-1) of *Plasmodium falciparum* are known to be protective against malaria. MSP-1 consists of a complex of four major non-covalently bound polypeptides; p30, p38, p42 and p83. In addition, MSP-1 complex proteins associate with MSP-6 and MSP-7 and form a functional complex in mediating some functional activity. Studies of the MSP-1 sequence obtained from different *P. falciparum* isolates demonstrated the presence of several conserved regions but also major dimorphic portions belonging to either the K1 (f) or the MAD20 (d) allelic families. To date, a few studies have examined the humoral immune responses to the MSP-1 complex as a whole in humans. In this study, we investigated if antibody responses to the MSP-1 complex, MSP-6 and MSP-7 have any protective correlation in cerebral malaria patients. To address this, we utilized subjects recruited in Central India which included three categories: healthy controls (HC), mild malaria (MM) and cerebral malaria (CM) patients. Plasma collected at enrollment of subjects were tested in an ELISA assay using recombinant proteins of all the MSP-1 complex proteins, including the two dimorphic families (d and f), MSP-6 and MSP-7, for the presence of antibody responses. We observed differential prevalence of anti-MSP-1 complex antibodies depending on the antigen. For all the antigens, the levels of total IgG increased in the MM category compared to HC. However, the antibody levels in general declined in CM patients as compared to MM and this reduction was significant for anti-MSP-1₁₈₃ (p<0.05). The predominant subclasses of IgG observed were IgG1 and IgG3 with negligible amounts of IgG2 and IgG4 for most subunits. We observed an increase in the prevalence and levels of both IgG1 and IgG3 in MM patients as compared to HC. In CM patients, IgG1 and IgG3 antibody levels were generally reduced compared to MM reaching significant reductions for anti-MSP-1₁₈₃ and MSP-1₁₈₃ IgG3 (p<0.05). These results suggest that there may be a deficit in the generation of optimal antibody response to some of the MSP-1 protein complex that may be relevant for protection in CM patients.

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**Sequence analysis of the apical membrane antigen 1 (AMA-1) of
Plasmodium vivax in Sri Lankan isolates**

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Abstract

Population diversity studies that analyze individual parasite antigens provide useful information on the impact of genetic variation on vaccine efficacy. Diversifying selection was previously observed strongly in Domain II of the gene encoding *Plasmodium vivax* apical membrane antigen-1 (PvAMA1) in Sri Lanka. Therefore, Domain II of PvAMA-1 was sequenced to examine population diversity both within and between geographical areas. Sequences were obtained for 37 single clonal Sri Lankan field isolates, and these were compared with other available Domain II PvAMA1 sequences (Sal-I, 11 Indian isolates, one each from the Philippines [PH84] and from Brisbane [PVQ]).

Analysis and comparison in a 411bp region of the Sri Lankan isolates at the nucleotide level showed 17 point mutations as against 9 reported previously. Further, 9 new non-synonymous replacements and 12 polymorphic sites were distinctive to the Sri Lankan isolates only. Three polymorphic sites seen in PH84, PV4 and PV12 were not found in our study.

Position 340 was the only amino acid position where all the Sri Lankan isolates were similar to PH84, PVQ and Sal-I. Similarly, two Sri Lankan isolates differed separately at positions 328 and 329, but rest of the Sri Lankan and Indian samples (N=46) were similar to PH84, PVQ and Sal-I. At position 310, a new amino acid change was observed in 4 isolates (Glutamate instead of a dimorphic change reported previously from Asparagine to Lysine). Comparison of the 18 haplotypes observed in the Sri Lankan isolates, revealed 7 isolates similar to Sal-I and one similar to an Indian isolate. Sixteen distinct haplotypes were identified from the Sri Lankan isolates that are reported for the first time.

Among the different *P. vivax* isolates, the PvAMA-1 gene exhibits sequence diversity and may be used as a marker for typing *P. vivax* populations. Based on these sequence variations, this polymorphic region could have significant effects on the immunological properties of the antigen.

Financial support by NSF/RG/2005/HS/06 and NRC Grant 05-34 are acknowledged.

011/A

Analysis of genetic polymorphism of *Plasmodium vivax* Duffy Binding Protein ligand domain of Sri Lankan isolates

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Interaction of *Plasmodium vivax* Duffy Binding Protein region II (PvDBPII) critical binding motif (CBM) with its erythrocyte receptor is critical for maintaining blood stage infections, rendering PvDBP a leading vaccine candidate. Since the efficacy of a vaccine based on a polymorphic antigen, such as PvDBP, is influenced by the local host immune response, characterization of the GD among local parasite strains is important in specific geographic settings. GD of the PvDBPII-CBM was assessed for the first time among field isolates from Sri Lanka. Forty single clonal *P. vivax* infections identified from two malaria endemic areas (Anuradhapura and Kataragama) and from a non-endemic area (Colombo), were used to generate nucleotide sequence data of CBM of PvDBPII (aa 285 to 521) by nested PCR amplification followed by direct sequencing.

Twenty dimorphic sites, 20 nucleotide polymorphisms and 15 haplotypes (haplotype diversity = 0.890) were identified at the CBM of PvDBPII compared to Sal-1 sequence. Genetic polymorphism in terms of pair wise diversity (π) and Tamura's three parameter model (d) were calculated to be 0.00950 (S.D.=0.00072) and 0.00959 (S.D.= 0.00052), respectively, consistent with published data from world wide isolates. Eighteen non-synonymous(NS) and 02 synonymous(S) mutations were identified, and the ratio of NS (0.01086) to S (0.00458) mutation rates was significantly >1, suggesting that positive selection acts on the CBS of PvDBPII. Residues essential for erythrocyte binding on PvDBPII-CBR were conserved in these 40 isolates. Six polymorphic residues recorded in high frequencies in worldwide isolates were also present among Sri Lankan isolates. Polymorphisms occurring at higher frequencies than Papua New Guinea isolates, of three amino acid residues involved in resistance to binding inhibitory antibodies were also detected.

Thus, even under low and unstable transmission conditions prevalent in the island, relatively high allelic diversity and positive selection acting on CBM of PvDBPII, possibly due to immune pressure were detected in Sri Lankan *P. vivax* field isolates.

Financial support by the National Science Foundation (Grant numbers NSF/SCH/2004/07 and NSF/RG/2005/HS/06) and the National Research Council (Grant numbers 05-34) is acknowledged.

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105/A

Genetic diversity in the *Plasmodium vivax* C-terminal 42 kDa region of merozoite surface protein-1(PvMSP-1p42) in Sri Lanka

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Proteolytic processing of *Plasmodium vivax* Merozoite Surface Protein-1 generates smaller fragments (i.e. 19 and 42 kDa fragments) which play a vital role during merozoite invasion. The high polymorphism observed in PvMSP-1p42 fragment is maintained by positive selection. We investigated the sequence polymorphism occurring in PvMSP-1p42 (p33 and p19) of 95 *P. vivax* infected isolates from three study areas, two endemic for malaria, Anuradhapura (N=22) and Kataragama (N=36) and one non-endemic area, Colombo (N=37) and carried out population genetic analysis with previously published geographically different field isolates (i.e. India, Bangladesh, Thailand, Brazil). Nucleotide diversity (π) for MSP-1p42 and p33 was high and similar to those reported from other geographic areas while though conserved, comparatively higher π value was obtained for p19 from Sri Lanka. The difference between rates of non-synonymous (dN) to synonymous (dS) substitutions indicated positive selection acting on p42 and p33 whereas purifying selection was acting upon p19 in Sri Lankan isolates. McDonald-Kreitman test for neutrality using *P. cynomolgi* as the out group verified this fact. Linkage disequilibrium index (R^2) plotted against nucleotide distances indicated intragenic recombination contributing to the increased diversity observed in p42. Our 95 sequences were 100% similar at the p19 fragment to the Sal I strain while a hypervariable region was clustered in the p33 fragment with an excess of non-synonymous amino acid polymorphisms. Further, Sal I was the most frequent phenotype observed from the Sri Lankan isolates compared to Belem phenotype observed from Thailand and Brazil. We obtained 27 amino acid haplotypes of which all were recombinants of the Sal I and Belem strains. Of these, 19 were novel haplotypes reported for the first time in this study. The phylogenetic tree revealed local isolates clustered among those from different countries. The PvMSP-1p42 gene signifies high genetic diversity in our island where unstable malaria persists with low transmission. The highly conserved p19 fragment gives an insight for a vaccine strategy based against *P. vivax* infections in geographically endemic regions including Sri Lanka.

Key Words: Sri Lanka, *Plasmodium vivax*, Genetic Diversity, Merozoite Surface Protein-1, Evolutionary biology

Acknowledgements: Financial support by the National Science Foundation (NSF/RG/2005/HS/06) and the National Research Council (05-34) is acknowledged.

B2 - Characterization of anti-PvDBPII binding inhibitory antibodies of *Plasmodium vivax* malaria patients from Sri Lanka

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The invasion of human RBCs by *Plasmodium vivax* depends on an interaction between the parasite Duffy binding protein (DBP) and the Duffy blood group antigen (DA) on RBCs, providing a unique opportunity for the development of an effective vaccination strategy based on DBP. Serum samples from acute vivax malaria patients that previously tested positive for total (IgG+IgM) anti-PvDBPII antibodies, from two malaria endemic areas (EA), Anuradhapura (N=41) and Kataragama (N=45), and non-endemic Colombo (NEA) (N=58) were used in an ELISA based binding inhibition assay to characterize the naturally acquired anti-PvDBP binding inhibitory antibodies (BIA).

Serum samples from all test areas contained anti-PvDBPII BIAs that exhibited inhibition at serum dilutions ranging from 1:5 to 1:100 in a dose dependent manner. 57%, 39% and 42% of samples from Colombo, Anuradhapura and Kataragama, respectively, possessed anti-PvDBPII BIAs that can specifically and effectively inhibit invasion at least up to 50%. While 4% of serum samples each from Colombo and Kataragama, and 5% from Anuradhapura demonstrated a high level of binding inhibitory activity with >90% inhibition capacity, 4% and 2% of the serum samples from these respective areas showed the same level of inhibition at a serum dilution of 1:10.

Magnitudes of IgM, IgG, IgG1 and IgG3 anti-PvDBPII antibodies significantly correlated with end point titers of BIAs (Spearman's correlation coefficient; $P < 0.05$) in NEA, but not in the two EAs ($P > 0.05$).

This study for the first time demonstrates functionality in terms of binding inhibition of naturally acquired effective antibodies to a putative erythrocytic stage vaccine candidate, DBP, existing even under low and unstable malaria conditions prevalent in Sri Lanka.

(Financial support by the National Science Foundation of Sri Lanka (grant numbers NSF/RG/2005/HS/06 and NSF/SCH/2004/07) and National Research Council (grant number NRC05-34) is acknowledged.)

B8 - Reactivity of synthetic peptides of the *Plasmodium vivax* Apical Membrane Antigen-1 (PvAMA-1) domain II with natural antibodies in sera of *P. vivax* patients from Sri Lanka

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Identification of T and B cell epitopes are of potential value in the development of a malaria subunit vaccine formulation. We previously demonstrated domain II of PvAMA-1 to be significantly diverse among Sri Lankan *Plasmodium vivax* field isolates. Hence to study the natural antibody response to PvAMA-1 domain II, seven overlapping peptides (P01-P07) were designed. While P04 and P05 were conserved in all isolates the remaining peptides contained polymorphic residues. These peptides were used in separate Indirect microplate ELISAs to assay the total (IgM+IgG) anti-PvAMA-1 antibody responses in serum samples from patients acutely symptomatic for *P. vivax* from the two malaria endemic areas Anuradhapura (N=39) and Kataragama (N=67) and from Colombo (N=56), a non endemic area for malaria.

While antibodies (IgM+IgG) to P04 and P05 recorded the highest prevalence among the three study areas, P03 and P06 documented the lowest (χ^2 test, $P<0.05$). Significantly high antibody prevalence to P03, P07 and P05 from Anuradhapura, and to P04 from Kataragama were apparent (χ^2 test, $P<0.05$). The antibody magnitude was highest for P07 in all test areas (Kruskal-Wallis test, $P<0.05$). A significant positive correlation between P04/P05, and P04/P07 antibody magnitudes among patients from Colombo, and P04/P05, P04/P07, P02/P07 from Kataragama were evident (Spearman correlation, $P<0.05$). Total antibody response showed a positive correlations with the number of past infections with P05 from Anuradhapura, parasitemia with P03 and age with P06 among residents of Kataragama ($P<0.05$).

In conclusion conserved epitopes, P04 and P05, with dominant linear B cell epitopes, had highest reactivity in all three test areas, therefore seems to be more immunogenic, while the others carrying diverse epitopes were less immunogenic. Further, it is imperative to carry out isotyping of the individual antibody responses to the seven peptides for in depth analysis.

(Financial support by the National Science Foundation (NSF/RG/2005/HIS/06) and the National Research Council (05-34) is acknowledged.)

***In vitro* plant regeneration of indica rice varieties: Bg-360 and Bg-250**

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Rice is the single most important crop in Sri Lanka which needs to improve the yield in order to combat with demands of increasing population. The improvement can possibly be achieved by genetic manipulation and it is highly dependent on the use of *in vitro* techniques. Callus induction and plant regeneration are prerequisites in *in vitro* techniques for the use of biotechnological approaches for rice improvement through genetic transformation. In this study the callus induction and plant regeneration were done with Bg-360, Bg-250 Sri Lankan rice varieties on modified N₆B₅ medium. High variability in callus induction and plant regeneration was observed among the cultivars. The candidate variety Bg-250 showed highest (90%) callus induction frequency than that of the other candidate variety Bg-360 (41.25%). Highly significant regeneration difference was observed in partially desiccated calli of Bg-360 in comparison to non-desiccated Bg 360 calli. It took 5-6 weeks for callus to regenerate into complete plants. The Bg-360 produced 16 and Bg-250 produced 13 plantlets per callus, with the regeneration frequencies of 42.42% and 95% respectively. The shoots regenerated from calli were successfully transferred to rooting medium for one week and plantlets with healthy roots were established in the soil. The results suggested two rice varieties used, Bg 250 showed the highest regeneration efficiency and after desiccation Bg 360 could successfully regenerated into complete plant. In conclusion, with standardized rice regeneration protocol, Bg 360 and Bg 250 are good candidates to transform with Arabidopsis STH2 gene in order to improve their yield.

Interallelic and intragenic recombination at the hypervariable region of merozoite surface protein-1 (MSP-1₃₃) of *Plasmodium vivax* in Sri Lanka

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Extensive polymorphism in *Plasmodium* Merozoite Surface Protein-1₄₂, a leading vaccine candidate for malaria, poses serious problems for vaccine development. Ninety five *P. vivax* isolates were investigated at the *Pvmsp-1₄₂* locus from malaria endemic and non-endemic sites in Sri Lanka. All 95 sequences were identical at the amino acid (a.a.) level of PvMSP-1₁₉, while 39 amino acid (a.a.) variant positions in PvMSP-1₃₃ defined 27 a.a. haplotypes with 19 unique to the island. Regions I (RI) and III (RIII) that flank the hypervariable region (RII) of PvMSP-1₃₃ included 2 and 1 unique variant positions, respectively. While, 24 of the 27 PvMSP-1₄₂ haplotypes represented 7 basic a.a. sequence types (HVR T1-T7) in the hypervariable region, the remaining 3 haplotypes, generated by interallelic double recombination between T1/T3 and T2/T3, represented HVR T8-T9 and HVR T10, respectively. A further 107 local and global isolates manifested 62 more a.a. haplotypes (H28-H89); 74 of these showed 9 of the 10 HVR types, with the exception of HVR-T7, unique to Sri Lanka. Two novel HVR types, T11 and T12, with a double recombination between HVR-T1/T3 and HVRT6/T2, are reported for the first time from South America and

Thailand, respectively. Intragenic recombination was a critical factor for the manifestation of a.a. haplotypes representing HVR-T3 to T7, and for the generation of H71-H89. In conclusion, under low transmission and unstable malaria conditions prevalent in the island, both interallelic and intragenic recombination appear to be critical for the origin of new PvMSP-1₄₂ amino acid haplotypes in local *P. vivax* populations.

Acknowledgement

Financial support by the National Science Foundation (NSF/RG/2005/HS/06) and the National Research Council (05-34) is acknowledged.

Phylogenetic relationship of *Plasmodium vivax* duffy binding protein gene between Sri Lankan and world wide isolates

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Phylogenetic relationship of the region II of the *P. vivax* Duffy Binding Protein gene (*PvdbpII*), a prime vaccine candidate against blood stage of vivax malaria, was analysed between parasite isolates from Sri Lanka and from different geographical areas, worldwide. A gene tree was derived from the aligned nucleotide sequences of a 672 bp region, using the Neighbour-Joining method with 1000 bootstrap replicates, the Tamura's three-parameter distance model as implemented in the MEGA version 4.0 programme. One hundred and seventy five *PvdbpII* sequences found in the GenBank were compiled and compared to 100 Sri Lankan (SL) sequences. The worldwide sequences included those from the reference Salvador-I strain, single sequences each from Vietnam (VIAT), Indonesia (INDO) and India (INDI) and 13, 17, 30 and 111 sequences from South Korea (SK), Colombia (COL), Thailand (Thai) and Papua New Guinea (PNG), respectively.

Twenty three different groups in all were evident in the gene tree. SL isolates were grouped in 6 different clades (A-G). A majority of these concentrated amongst 3 different clades (A, B and C), with no clustering detected according to the sample collection sites within the country. A single clade (D), exclusively contained SL isolates, proving evidence for geographical isolation. Two other clades (A & F) contained isolates from SL in combination with THAI and SK, respectively. The close phylogeny of the SL sequences with world wide ones were more clearly evident in the gene tree by SL isolates grouping with those from PNG, THAI, COL, INDO and VIAT in clade B, and with those from PNG, THAI, COL and from INDI with the reference strain Sal-1 in clade E. Interestingly, both these groups lacked SK isolates. The Sri Lankan *PvdbpII* sequences thus appeared to represent a sample from the *PvdbpII* worldwide genetic diversity, rather than from any particular lineage.

Acknowledgement

Financial support by the National Science Foundation (NSF/RG/2005/HS/06, NSF/SCH/2004/07) and the National Research Council (05-34) is acknowledged.

B2 - Comparison of antibody responses to asexual stage vaccine candidate antigens of *Plasmodium vivax* Merozoite Surface Protein-1 (MSP-1) and Apical Membrane Antigen-1 (AMA-1) in Sri Lanka

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Lack of amino acid polymorphism at the domain II loop of PvAMA-1(PvAMA-1DIIL) and PvMSP-1₁₉, and the “protective” antibody responses targeting these two antigens, render these suitable to be included in an asexual stage multi-component vaccine against *Plasmodium vivax* in Sri Lanka. Hence, the naturally acquired antibody responses (IgM+IgG) against PvAMA-1DIIL and PvMSP-1₁₉ of *P. vivax* infected patients from a non-endemic area, Colombo (N=37) and two malaria endemic areas, Anuradhapura (N=35) and Kataragama (N=61) were compared.

A significantly higher proportion of serum samples (67%) from Kataragama recognized both PvAMA-1DIIL and PvMSP-1₁₉ antigens (χ^2 , $P<0.05$) compared with those from Colombo (51%) and Anuradhapura (32%), while 43%, 52% and 33% from Colombo, Anuradhapura and Kataragama, respectively, recognized either one of the two antigens. Nonetheless, responders from Colombo and Kataragama recognized both antigens compared to preferential recognition to each separately. Anuradhapura responders tend to recognize PvMSP-1₁₉ more (χ^2 , $P<0.05$). Responders to both antigens in Colombo and Kataragama recorded a positive correlation between PvAMA-1DIIL and PvMSP-1₁₉ (Pearson correlation, $P<0.05$) while a negative correlation was observed in Anuradhapura. With increasing exposure to malaria, the antibody prevalence of individuals who recognized both the antigens increased in Kataragama (Chi square for linear trend, $P<0.05$), where the isotype switch from primary IgM to cytophilic protective IgG was documented for each antigen in our previous studies.

Thus, the suitability of PvAMA-1DII and PvMSP-1₁₉ to be included in a multi-component vaccine is clearly evident.

(Financial support by the National Science Foundation (NSF/RG/2005/HS/06) and the National Research Council (05-34) is acknowledged.)

1 - Strain specific and strain transcending antibody responses directed against *Plasmodium vivax* Duffy Binding Protein II (PvDBPII) in Sri Lanka

1 Masad H Premaratne, Preethi V Udagama-Randeniya

Department of Zoology, Faculty of Science, University of Colombo, Colombo

The strain dependant nature of the naturally acquired (NA) immunity is a major challenge to develop effective vaccine(s) against malaria. Attempts were made to ascertain correlations between NA antibody responses against *P. vivax* Duffy Binding Protein II (PvDBPII), a leading blood stage vaccine candidate against vivax malaria, with the homologous PvDBPII amino acid (a.a.) haplotypes, in Sri Lanka. *P. vivax* DNA samples (N=100) were used to derive PvDBPII haplotypes while the homologous serum samples were assayed by ELISA against PvRPII (recombinant protein based on reference PvDBPII Sal-1 strain) for specific total (IgG+IgM), IgG, and binding inhibitory antibodies (BIAs).

Percentage a.a. homology to Sal-1 strain of the 5 most prevalent haplotypes (SL-1 to SL-5) locally, were 98.66%, 97.32%, 98.21%, 96.42% and 95.98%, respectively, while prevalence of specific homologous total antibodies were 62%, 69%, 87%, 83% and 100%, with corresponding IgG prevalences of 92%, 82%, 69%, 80% and 40%, respectively. Among local PvDBPII haplotypes, 62% were able to induce NA BIAs with the ability to block the specific binding between the PvRPII and its erythrocyte receptor. Anti-PvRPII BIA titers showed an association with the percentage a.a. homology of the haplotypes to the reference. Serum samples of the PvDBPII SL-1 contained BIA's up to 1:100 dilutions, while those of SL-3 and SL-6 (with equal homology) had BIAs up to 1:5 and 1:50, respectively. SL-5 with lowest percentage homology contained BIA's up to the lowest serum dilution (1:5). Thus naturally existing PvDBPII haplotypes mount both strain specific and strain transcending anti-PvDBPII NA antibody responses.

(Financial support by the National Science Foundation - Grant numbers NSF/SCH/2004/07 and NSF/RG/2005/HS/06, and the National Research Council (05-34) is acknowledged)

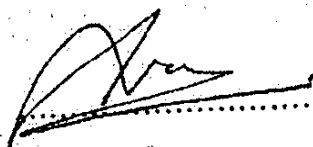
Research Management Unit,
No: 94, Cumaratunga Munidasa Mawatha,
University of Colombo.
Colombo - 03.

22nd April 2010

Accountant,
National Science Foundation,
No: 47/5,
Maitland Place,
Colombo -07.

Statement of Receipts & Payments - RG/2005/HS/06

Please find enclosed here with a statement of Receipts & Payments of the Research Grant
No: RG/2005/HS/06 for the period ended 22.04.2010



S M S S Nagahapitiya,
Senior Assistant Bursar,
Research Management Unit,
University of Colombo.

Statement of Variance Analysis as at 22.04.2010

INT NO: RG/2005/HS/06

Item	Approved Amount	Actual Payment	Balance
Grants	1,376,000.00	1,381,723.25	(5,723.25)
Miscellaneous	64,500.00	56,500.00	8,000.00
Equipment	100,000.00	102,276.75	(2,276.75)
Total Amount	1,540,500.00	1,540,500.00	-

Funds received from National Science Foundation

1,540,500.00

Total Payment as at 22.04.2010

1,540,500.00

Balance as at 22.04.2010

-

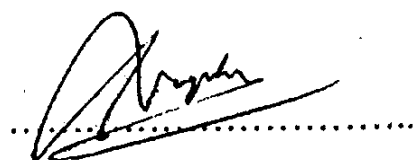


S M S S Nagahapitiya,
Senior Assistant Bursar,
Research Management Unit,
University of Colombo.

Cc : Dr. Preethi Randeniya/Department of Zoology

Statements of Receipts & Payments for the period ended 22.04.2010
GRANT NO: RG/2005/HS/06

	<u>Rs.</u>	<u>Rs.</u>
<u>Receipts</u>		
Cheque # 543998-22.07.2005	472,500.00	
Cheque # 554677-27.09.2005	3,000.00	
Cheque # 568926-30.12.2005	4,750.00	
Cheque # 674317- 12.07.2006	396,000.00	
Cheque # 532702-12.10.2006	5,000.00	
Cheque # 545163-17.04.2007	10,000.00	
Cheque # 579957-16.04.2007	109,750.00	
Cheque # 583809-14.06.2007	495,000.00	
Cheque # 870360-24.07.2008	20,000.00	
Cheque # 426190-16.01.2008	10,000.00	
Cheque # 426579-07.05.2008	14,500.00	
		1,540,500.00
<u>Payments</u>		
Consumables	1,381,723.25	
Miscellaneous	56,500.00	
Equipment	102,276.75	1,540,500.00
		1,540,500.00
Funds received from National Science Foundation		1,540,500.00
Total Payment as at 22.04.2010		1,540,500.00
Balance as at 22.04.2010		



S M S Nagahapitiya,
Senior Assistant Bursar,
Research Management Unit,
University of Colombo.

Cc : Dr. Preethi Randeniya/Department of Zoology

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