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

A thesis submitted for the Degree of Doctor of Philosophy

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Dedicated to my dearest
Amma & Thaththa

Declaration

This thesis is my original work and has not been submitted previously for a degree at this and any other university / institute. To the best of my knowledge it does not contain any material published or written by another person, except as acknowledged in the text.

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Abstract

The efficacy of a vaccine is hindered by the antigenic polymorphism elicited by the parasite to escape the host immune system. Thus, the present study for the first time characterized the prevailing genetic diversity of two major asexual stage putative vaccine antigens of *Plasmodium vivax*, the Apical Membrane Antigen-1 domain II (*ama-1DII*) and the Merozoite Surface Protein-1_{42} (*msp-1_{42}*) in Sri Lanka, where unstable malaria prevails with low transmission. Parasite isolates (N=217) were collected from patients infected with *P. vivax* malaria from two malaria endemic areas, Anuradhapura and Kataragama, and from non-endemic Colombo. The selected single clone isolates (N=169) identified by a combined polymerase chain reaction/restriction fragment length polymorphism (PCR/RFLP) of the *Pvmsp-3α* locus were amplified at the *Pvama-1DII* and *Pvmsp-1_{42}* locus by the standard PCR technique. Both the local and the worldwide genetic diversity of these two antigens were analysed.

The *ama-1DII* gene in Sri Lanka showed meagre meiotic recombination with the enclosure of single nucleotide polymorphisms (SNPs), where eleven amino acid (a.a) variant positions defined 21 a.a. haplotypes with 10 unique to Sri Lanka. The H1 a.a. haplotype predominant in Sri Lanka was identical to the reference Salvador I strain, while haplotypes H14 and H16 were observed in Sri Lanka, India and Venezuela. Further, 145 globally dispersed isolates defined 12 a.a. haplotypes (H22-H33), where 7 and 1 haplotypes were unique to India and Venezuela, respectively. A novel observation where all a.a. sequences locally and globally were identical at the domain II loop of PvAMA-1 was recorded. The evolutionary relationship in the phylogenetic tree revealed fewer clustering where most isolates had a very recent common origin. Evidence of reduced genetic diversity in Venezuela reflected the effects of recent spread of the parasite to the new world, whereas those from the old world appeared to reflect a very ancient selectively maintained polymorphism.

At the PvMSP-1_{19} locus, all a.a. sequences were identical. The analysis of 39 variant a.a. positions upstream of PvMSP-1_{19}, at the PvMSP-1_{33} region, documented several novel observations: i) this region defined 27 a.a. haplotypes with 19 unique to Sri Lanka, ii) 24 of the 27 PvMSP-1_{42} haplotypes represented 7 basic a.a. sequence types at the hypervariable region (HVR) while the remaining 3 were generated by interallelic double recombination, iii) sequences from widely dispersed isolates in the database defined 62 more a.a. haplotypes, 43 of which corresponded to 9 of the 10 HVR types (excluding HVR-T7, unique to Sri Lanka), iv) two novel HVR types, HVR T11 and T12, with a double recombination were derived from South America and Thailand, respectively, and v) intragenic recombination accounted for a.a. haplotypes HVR-T3 to T7, and for the generation of H71-H89. T cell epitope polymorphism arising due to non-synonymous substitutions at PvMSP-1_{33} may result in differential binding of the polymorphic peptides to class II MHC alleles, and may evoke different host immune responses. Hence, the extensive allelic polymorphism evident at *Pvmsp-1_{33}* was due to recombination, mutation and positive selection.

This study for the first time reported 33 combined amino acid haplotypes that were generated by the PvAMA-1DII and the PvMSP-1_{33} amino acid sequences that demonstrated the extensive allelic polymorphism present in Sri Lanka.

Subsequently, to evaluate the ensuing strain-specific immunity arising due to extensive antigenic polymorphism, amino acid sequences of PvAMA-1DII were aligned with the homologous total (IgM+IgG) antibody responses assayed by an indirect enzyme linked immunosorbant assay (ELISA) established in-house against seven PvAMA-1DII synthetic peptides (P01-P07). This analysis was repeated for the a.a. sequences of PvMSP-$1_{42,}$ and PvMSP-1_{19} with the previously published antibody responses assayed against PvMSP-$1_{42,}$ and PvMSP-1_{19} recombinant proteins. A strain-transcending (cross reactive) immune response was clearly prevalent against peptide P07 of PvAMA-1DII and the PvMSP-1_{42} protein. Conversely, anti-P04peptide and anti-PvMSP-1_{19} antibody prevalence precluded strain-specific immune response against the domain II loop of PvAMA-1 and PvMSP-$1_{19,}$ respectively. More importantly, both these regions clearly elicited a protective antibody response where an isotype switch was observed from a primary IgM response to a functionally important cytophilic IgG with increasing exposure to malaria in endemic residents.

A multi-component vaccine containing a cocktail of parasite antigens would be the final goal of vaccine development against the asexual stages of *Plasmodium*. Thus, it is crucial to identify multiple protective domains or epitopes from different antigens that are protective against the natural malaria infections. Hence, this study signifies that the highly conserved domain II loop of PvAMA-1 and the 19 kDa fragment of PvMSP-1_{42} may be suitable as veritable vaccine components to develop "protective" immunity against *P. vivax*.

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Abbreviations

Ab – Antibody

ABRA – Acidic basic repeat antigen

ADCI – Antibody dependent cellular inhibition

AMA-1 - Apical membrane antigen-1

AMC – Anti malaria campaign

API – Annual parasite incidence

BSA - Bovine serum albumin

cAMP- Cyclic adenosine triphosphate

CB – Conserved blocks

CD4, 8, 25 – Cluster determinant factor 4, 8, 25

CDC - Center for disease control and prevention

CQ – Chloroquine

crt - Chloroquine resistance transporter

CSP – Circumsporozoite surface protein

DARC - Duffy antigen receptor for chemokines

DBL – Duffy binding like

DBP – Duffy binding protein

DDT - Dichlorodiphenyltrichloroethane

DEET - N, N-diethyl-meta-toluamide

DHFR - dihydrofolate reductase

DNA - Deoxyribonucleic acid

EDTA - Ethylenediaminetetraacetic acid

EGF - Epidermal growth factor

ELISA - Enzyme linked immunosorbant assay

ESMS - Electrospray mass spectrometry

FCA - Freund's complete adjuvant

F_{ST} – Fixation index

G-6PD - Glucose-6-phosphate dehydrogenase

GAP - Genetically attenuated parasite

GDP – Gross domestic product

GHA - General hospital of Anuradhapura

GIA – Growth inhibition assay

GPI - Glicosylphosphatidylinositol

H_2O_2 - Hydrogen peroxide

HLA – Human leukocyte allele

HRP - Horseradish peroxidase

HSBA - High specific binding activity

HVR – Hypervariable region

ICB - Interallele conserved blocks

IFA - Immuno fluorescent assay

IFNγ - Interferon gamma

IgG – Immunoglobulin G

IgM – Immunoglobulin M

IL12 - Interleukin 12

IRIV - Immunopotentiating reconstituted influenza virosome

IRS – Indoor residual spraying

LD - Linkage disequilibrium

LGS - Linkage group selection

Mab - monoclonal antibodies

$MgCl_2$ - Magnesium chloride

MHC - Major histocompatible complex

MRS - Malaria research station

MRU - Malaria research unit

MSP – Merozoite surface protein

MSP-1 – Merozoire surface protein-3

MSP-1 - Merozoite surface protein-1

NCBI - National center for biotechnology information

NET – Sodium EDTA

NF – Nuclease free

NHSL – National hospital of Sri Lanka

NI – Neutrality index

OPD - O-phenylenediamine

ORF – Open reading frame

PAN - *P*lasminogen, *A*pple, *N*ematode

PB – Phosphate buffer

PCR - Polymerase chain reaction

PE – Previously infected

PNE – Previously not infected

PNG - Papua New Guinea

PQ – Primaquine

RBC – Red blood cell

RBL- Reticulocye binding protein like protein

RBL – Reticulocyte binding like

RBM - Roll back malaria

RBP - Reticulocyte binding protein

RFLP - Restriction fragment length polymorphism

Rm – Recombination event

RP-HPLC - Reverse phase high performance liquid chromatography

Sal I – Salvador I allelic type of *P. vivax*

SERA - Serine repeat antigen

SNP - Single nucleotide polymorphism

SP – Sulphadoxine

TAE - Tris-Acidic acid-EDTA

Taq - Thermus aquaticus enzyme

TBI - Transmission blocking immunity

TBV - Transmission blocking vaccines

TE – Tris EDTA

TNF-α – Tumour necrosis factor-α

TRAP – Thrombospondin related adhesive protein

UIS – Up regulated in infectious Sporozoites

UV illuminator – Ultraviolet illuminator

CHAPTER 1

Introduction

1.1. World malaria status

Being one of the world's most prevalent parasitic diseases, Malaria 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). 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 (Breman *et al.* 2001), however currently the malaria burden has decreased in many parts of the world (World Malaria Report, 2010).

It is estimated that the number of cases of malaria rose from 233 million in 2000 to 244 million in 2005 but decreased to 225 million in 2009. The number of deaths due to malaria is estimated to have decreased from 985,000 in 2000 to 781,000 in 2009. Decreases in malaria burden have been observed in all WHO Regions, with the largest proportional decreases noted in the European Region, followed by the Region of Americas. The largest absolute decreases in deaths were observed in Africa. The fragility of malaria control and the need to maintain control programmes even if numbers of cases have been reduced substantially (World Malaria Report, 2010).

1.2. 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). Nonetheless, *P. vivax* is usually considered a "benign" infection, compared to *P. falciparum*, the burden of vivax malaria should not be under appreciated as it exacts a significant toll on 40% of the world's population (Price *et al.* 2007). 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* infection (Herrera *et al.* 2007). The number of people at risk of infection reaches 2.6 billion in population density areas endemic

for *P. vivax*, which is slightly greater than that for *P. falciparum* (Guerra *et al.* 2006). In South and Southeast Asia, where majority of *vivax* malaria occurs, accounts for up to 50% of malaria cases with prevalence rates between 1% and 6% of the population (Tjitra *et al.* 2005; Zhou *et al.* 2005) and in India it accounts for up to 65% of malaria cases (1http://malariavaccine.org/mal-what_is_malaria.htm). Frequent international travel, widespread presence of *Anopheles* mosquitoes, and environmental conditions that favour malaria transmission have caused *P. vivax* to occur with increasing frequency in non endemic areas.

Vivax malaria stands alone among the *Plasmodia* infecting humans in its capacity to reach well into the temperate latitudes, as it does today up to the Korean peninsula and across the southern temperate latitudes of Asia to the Mediterranean Sea (Baird, 2008). The proportional burden of *vivax* is even greater in Central and South America, reaching 71–81% of all malaria cases (Duarte *et al.* 2004). In America, *P. vivax* infection is present in 20 countries from the southern coasts of Mexico to the Brazilian forests, where it represents the most prevalent malaria species. Colombia together with Brazil produces majority of *vivax* malaria cases on the South American continent, and in Colombia alone it represents 65% of the clinical cases reported annually (Dutta *et al.* 2005a). In eastern and southern Africa alone, 5% of malaria infections are attributable to *P. vivax*, although this still accounts for between 6 and 15 million cases per year, where the Duffy receptor is lacking among much of these populations (Mendis *et al.* 2001).

1.3. Socio economic burden of vivax malaria

Malaria is a preventable infection that carries with it an enormous global burden. Principally, disease affecting poor people living on the margins of developing economies, vivax malaria traps many societies in a relentless cycle of poverty. Intermittent transmission makes protective immunity rare, and the disease strikes all ages. Repeated acute febrile episodes of debilitating intensity can occur for months. In children this can lead to life-long learning impairment, whereas incapacitation of adults has tremendous direct economic consequences through lost productivity and depletion of meagre financial reserves (Carlton *et al.* 2008).

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1 Fact sheet: *Plasmodium vivax* malaria. {http://malariavaccine.org/mal-what_is_malaria.htm}, 26.05.2009.

The majority of malaria cases and deaths occur in impoverished regions of the world in which malaria is endemic, and the disease poses a significant economic burden for these regions (Suh *et al.* 2004). Poor populations are at greatest risk; 58% of the cases occur in the poorest 20% of the world's population and these patients receive the worst care and have catastrophic economic consequences from their illness. This social vulnerability requires better understanding for improving deployment, access, quality, and use of effective interventions (Breman, *et al.* 2004). The disease is responsible for an estimated average annual reduction of 1.3% economic growth for those countries with the highest burden (World Malaria Report, 2005) and it costs the continent more than US$ 12 billion annually (2World Bank Malaria, 2001). Macroeconomic studies recorded that in highly endemic countries, malaria may be responsible for reducing economic growth by more than one percentage point a year (Malaney *et al.* 2004), intimately connecting malaria and poverty. Controlling for factors such as tropical location, colonial history, and geographical isolation, in 1995, countries with intensive malaria had income levels of only 33% of countries without malaria (Gallup and Sachs, 2001). Macroeconomic projections show that the costs are far greater than the costs of individual cases, with a substantial deleterious impact of malaria on schooling of patients, external investments into endemic countries, and tourism.

Malaria risk has always been geographically specific. Intensive malaria is confined to the tropical and subtropical zone where poor countries predominate. Almost all of the rich countries are outside the bounds of intensive malaria (Gallup and Sachs, 2001). Despite the fact that strong attempts to eradicate malaria have been made, the disease burden is still on the rise and some estimate that the number of cases could double in the next twenty years if development of new methods for control is not established (Rogerson & Carter 2008). Aside from human tragedy, an economic disaster is also likely predicted for malaria stricken countries. Gallup and Sachs (2001) report that, during 1965 - 1990, the annual growth rates in malarious countries were 1.3 % lower compared to non-malarious countries. This corresponds to a 50% decrease in per capita Gross Domestic Product (GDP) (Gardner *et al.* 2002). Sachs & Malaney (2001) hypothesized that this apparent correlation between poverty and malaria runs both ways. Poverty may increase the incidence of malaria; malaria may also increase the likelihood of poverty. An increase in population in malarious regions compounded by weak public health systems in developing countries climate changes (Hay *et*

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2 The World Bank. Malaria-at-a-glance. March 2001.{www1.worldbank.org/hnp/Malaria/index.asp}, 06.08.2009.

al. 2002), new agriculture practices such as irrigation and dam construction (Sachs & Malaney, 2002), increased resistance to antimalarial treatments and insecticides (Bozdech *et al.* 2003) and the complexity and flexibility of the host genetics (Gardner *et al.* 2002) have all contributed to the increase in malaria.

The socio-economic burden of *P. vivax* infection is unknown. Based on the estimated number of clinical episodes per year (106–313 million), assuming full treatment coverage with drug costs of $0.1 per course of Chloroquine (CQ) and non-drug treatment costs of US$ 8.3 (3WHO CHOICE study, 2007), the direct cost of treatment is between US$ 0.9 and 2.7 billion per year. As each symptomatic infection is associated with at least 3 days of absence from school or work, the overall global cost of *P. vivax* infection to the individual in terms of lost productivity, healthcare costs and transport to clinics can be conservatively estimated as being between US$ 1.4 and 4.0 billion per year. These immediate costs are likely to be augmented by recurrent *P. vivax* infections in children. Longitudinal studies from Sri Lanka suggest that repeated attacks of malaria have an adverse impact on the school performance of children (Fernando *et al.* 2003a & 2003b). Intuitively, recurrent vivax malaria will also result in anaemia, malnutrition, growth retardation, and impaired economic productivity, though currently detailed studies are unavailable. The burden of vivax malaria is felt not only at the individual level but also by healthcare providers. In many parts of Asia and South America, where *P. vivax* accounts for more than half of the cases of malaria, institutional healthcare costs are appreciably higher due to high rates of clinical attendance and hospitalization. In Thailand and Indonesia almost 30% of patients hospitalized with malaria are associated with pure *P. vivax* infections (Tjitra *et al.* 2005; Carrara, *et al.* 2006).

1.4. 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 the World Health Organization (WHO) reports (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 in the island and the annual economic loss due to malaria in Sri Lanka

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3 WHO-CHOICE Study. Mean cost of health centre consultation cost for WPR B and SEAR B regions. {http://www.who.int/choice/costs/unit_costs/en/index.html}, 20.12.2007.

has been estimated at US$ 15.5 million comprising a 6% loss of annual income (Wickremasinghe, 2002).

In Sri Lanka, 88% of cases were accounted by the malaria species *P. vivax* (Annual Health Bulletin, 2002). According to the Anti malarial campaign (AMC), from 2000 until 2007 a dramatic decrease in *P. vivax* cases were recorded in Sri Lanka, however, in 2008, of the 670 malaria positive cases, 623 individuals were positive for *P. vivax* (93%) (Galappaththy, G. N. L., Personal communication). Estimating the prevalence of *P. vivax* in districts of Sri Lanka revealed that in 2008 the highest number of *P. vivax* cases was reported from Killinochchi (402 infected out of 670 cases), while 71, 48 and 46 cases were reported from Vavuniya, Mannar and Mullativu, respectively.

The annual parasite incidence (API) of *P. vivax* malaria cases at medical officer of health (MOH) areas had marked variation throughout the island, where districts of Jaffna, Kilinochchi and Mullaitivu in the north, Monaragala and the South Eastern MOH areas in Ratnapura district showed high malaria incidences. The API of *P. falciparum* and mixed infections was generally much lower than the API of *P. vivax*, although the spatial distribution is somewhat similar (Galappaththy, G. N. L., Personal communication). In 2004, Pollonnaruwa recorded the highest API per 1000 of >2.1, while the western and southern parts of Sri Lanka and Killinochchi, Mullaitivu, Puttalam, Kandy, Nuwaraeliya had an API per 1000 of 0-0.5. However, during the following years these API restricted districts changed, where, Colombo, Gampaha, Kaluthara, Nuwaraeliya, Matale were the only districts representing an API per 1000 of 0-0.009 in 2008, while, Killinochci represented an API per 1000 of ≥1 and Mullaitivu represented an API per 1000 of 0.1-0.5. Majority of the districts in Sri Lanka had an API per 1000 of 0.01-0.05 (Galappaththy, G. N. L., Personal communication).

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. The 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. 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).

At district resolution, over the period of January 1995 to December 2000, monthly parasite incidence of *P. falciparum* and *P. vivax* malaria were 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 may be due 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).

1.4.1. Vectors of malaria in Sri Lanka

Anopheles culicifacies was found to be the main vector, which breeds mainly in pools in stagnant rivers, and therefore, its density is mostly dependent on temporal and spatial variations in rainfall and river flow (reviewed in Konradsen *et al.* 2000; Surendra *et al.* 2003). The secondary vectors (*An. subpictus* and *An. annularis*) play only an insignificant role in malaria transmission in Sri Lanka (Annual Health Bulletin, 2002). Twenty two *anopheline* species are reported to date as potential vectors in Sri Lanka (Reviewed in Konradsen *et al.* 2000). In the intermediate zone of the country, Ramasamy and others (1994) found the peak biting time for *An. Culcifacies* to be during the night. In general it is believed that *An. culcifacies* bites outdoors as well as indoors, however, Herath and Joshi (1989), and Dissanaike (1984) report that *An. culicifacies* mainly resides inside houses and sheds after taking a blood meal (reviewed in Konradsen *et al.* 2000). A series of mark-release recapture experiments on *An. Culicifacies* demonstrated that this species could travel at least 500 m in one night and that 2–7% of *An. culicifacies* mosquitoes had flown a distance of 2 km to a recapture village within 4–7 days of marking and release. Although the estimation of flight ranges has a number of methodological difficulties and depends on the local ecology, it is expected that people living close to the breeding sites would be at higher risk for the disease (Reviewed in ven der Hoek *et al.* 2003).

1.4.2. Vector resistance in Sri Lanka

Control of malaria in Sri Lanka is primarily through the use of insecticides. Prior to 1975/77, malaria vector control programmes were based on indoor residual house spraying (IRS) of dichlorodiphenyltrichloroethane (DDT). In 1969, the first report of resistance to DDT by *An. culicifacies* was reported (Pinikahana & Dixon, 1993) and due to development of resistance to DDT in vector mosquito populations and environmental concerns, DDT was replaced with malathion in 1975 (Konradsen *et al.* 2000). However, the vector resistance to Malathion, and increased transmission of malaria, lead to the introduction of pyrethroids for vector control programmes in Sri Lanka in 1994 (Perera *et al.* 2008). The status of insecticide resistance and prevalence of different types of resistance mechanisms in *An. culicifacies* and *An. subpictus* populations from five administrative districts of Sri Lanka reported by Perera *et al.* (2008) showed that resistance to DDT and Melathion is common in all *anopheline* species in Sri Lanka except in one city for the latter.

Though, insects acquire target site insensitivity through point mutations, only a limited number of changes can decrease insecticide sensitivity without disrupting the normal physiological functions of the target site (Hemingway *et al.* 2004). These point mutations were observed on the target sites of DDT and pyrethroids of *An. subpictus* (Karunaratne *et al.* 2007). Further insensitivity of the carbamate and organophosphate target sites was detected in almost all the populations of *An. culicifacies* and *An. subpictus*. Heterogeneity of vector resistance to insecticides may also be due to the presence of sibling species with different insecticide cross-resistance spectra (Abhayawardana *et al.* 1996; Kelly-Hope *et al.* 2005). Currently, genomic diversity, differential up regulation, different binding specificities of resistance conforming genes, and the presence of different subspecies with varying vectorial capacities have imposed severe threats to malaria vector control programmes (Perera *et al.*, 2008).

1.4.3. Chemoprophylaxis of malaria in Sri Lanka

Chloroquine (CQ) plus primaquine (PQ), and sulphadoxine/pyrimethamine (SP) plus PQ are used as first and second line treatment, respectively, against uncomplicated malaria infections in Sri Lanka (Schousboe, *et al.* 2007). However, primaquine is not administered to children below the age of one and pregnant mothers and SP is not recommended in the last trimester of pregnancy, first six weeks of lactation and for children below two months of age (Briët *et al.*

2005). Those with known glucose-6-phosphate dehydrogenase (G-6PD) enzyme deficiency is at risk if primaquine is administered as it can cause serious destruction of red blood cells (haemolysis) (Konradsen *et al.* 2000). Primaquine is added to chloroquine treatment as it is active against the hypnozoite stage that occurs in *P. vivax* infections. This action against hypnozoites prevents relapses of *P. vivax*. This is useful in low transmission areas but not so much under conditions of intense transmission when re-infection is common. Primaquine also kills the sexual stages of *P. vivax*, however, there is no clear evidence that the effect of PQ on liver stages or gametocytes of *P. vivax* reduce transmission (Konradsen *et al.* 2000). The recommended treatment for *P. falciparum* malaria has been changed to artemisinin based combination therapy (ACT) (Rajakaruna *et al.*, 2008).

Thus far, *P. vivax* resistance to either CQ (Konradsen *et al.* 2000) or SP have not been recorded in Sri Lanka. A descriptive study analyzed sulphadoxine/pyrimethamine (SP) resistance-related single nucleotide polymorphisms (SNPs) in the *P. vivax* dihydrofolate reductase (*Pvdhfr*) and dihydropteroate synthase (*Pvdhps*) genes in samples originating from nine districts in Sri Lanka (Schousboe *et al.* 2007). The outcome demonstrated that the high frequency of mutant haplotypes related to pyrimethamine resistance was perturbing as it indicates that drug tolerant/resistant *P. vivax* parasites have evolved despite a low level of SP drug pressure, possibly attributed to the use of other antifolate drugs. It is unknown whether these mutant *P. vivax* haplotypes do exhibit SP resistance *in vivo*.

1.5. Life cycle of *Plasmodium*

The protozoan *Plasmodium* is transmitted to humans by mosquitoes of the genus *Anopheles*, where the mosquito picks up the parasite during a blood feeding from an animal with parasitaemia. *Plasmodium* parasites are protozoa of the phylum Apicomplexa (often referred to as sporozoans). These are animal parasites, which exist and survive in two hosts, having both sexual and asexual stages, alternating between haploid and diploid phases (Bozdech *et al.* 2003).

Infection in the human host begins when an infected female *Anopheles* mosquito feeds on a human host and injects both anticoagulant and haploid sporozoites into the human blood stream. Subsequently, within 30-60 minutes, the sporozoites travel to the hepatocytes (liver cells), where they mature. However, in *P. vivax* a proportion of the liver-stage parasites (hypnozoites) experience a dormant period instead of immediately undergoing an asexual

replication, where they reactivate several weeks to months (or years) after the primary infection which leads to relapses (Baton and Ranford-Cartwright, 2005). In the hepatocyte, the sporozoites undergo multiple asexual fissions, or exo-erythrocytic (or pre-erythrocytic) schizogony. Schizogony refers to a replicative process in which the parasite undergoes multiple rounds of nuclear division without cytoplasmic division followed by a budding, or segmentation, to produce thousands of infective, haploid progeny called merozoites (Kyes *et al.* 2001). Following rupture of the host hepatocyte, the merozoites are released to the blood stream where they rapidly adhere to host erythrocytes (red blood cells). The merozoites recognize specific proteins on the surface of the erythrocyte and actively invade the cell in a manner similar to other apicomplexan parasites (Baton and Ranford-Cartwright, 2005). These parasites have high metabolic demands and cannot survive in the compromised red blood cells (RBCs) of a human with sub-optimal oxygen carrying capacity. At this point, the patient is still in the pre-patent period and does not exhibit any clinical symptoms.

Subsequent to erythrocyte invasion, the parasite undergoes a trophic period followed by an asexual replication. The young trophozoite is often called a "ring form" due to its morphology in Geimsa-stained blood smears. As the parasite develops and matures into a trophozoite, it ingests the host cell cytoplasm and breaks down the haemoglobin into amino acids. Haemozoin, the by-product of the haemoglobin digestion forms golden-brown to black granules and have been long recognized as a distinctive feature of blood-stage parasites (Baton and Ranford-Cartwright, 2005). Nuclear division marks the end of the trophozoite stage and the beginning of the schizont stage. Erythrocytic schizogony consists of 3-5 rounds (depending on species) of nuclear replication followed by a budding process, where each merozoite gives rise to 6-32 daughter merozoites during 24-72 hours depending on the species (Kyes *et al.* 2001). The merozoites released with the rupturing of erythrocytes invade new erythrocytes and initiate another round of schizogony. The blood-stage parasites within a host usually undergo a synchronous schizogony. The simultaneous rupture of the infected erythrocytes and the concomitant release of antigens and waste products accounts for the hallmark clinical presentation of the disease, which corresponds to intermittent fever paroxysms associated with malaria (Baton and Ranford-Cartwright, 2005). This cycle continues until the patient dies or the parasite is slowed by either the host immune system or chemotherapeutic agents.

A small portion of newly invaded merozoites is sexually differentiated in to forms of male macrogametocytes or female microgametocytes. The factors involved in the induction of

gametocytogenesis are not known (Baton and Ranford-Cartwright, 2005). Gametocytes do not cause pathology in the human host and will disappear from the circulation if not taken up by a mosquito. The gametocyte's ingested by *Anopheline* mosquitoes form micro (female) and macro (male) gametes. After ingestion by the mosquito, the microgametocyte undergoes three rounds of nuclear replication. These eight nuclei then become associated with flagella that emerge from the body of the microgamete. This process is readily observable by light microscopy due to the thrashing flagella and is called ex-flagellation.

The highly mobile microgamete's will seek out and fuse with a macrogamete to form a diploid zygote which, within 12-24 hours develops into an oökinete, the only diploid stage during the lifetime of the *Plasmodium* parasite. The oökinete, which transverse both the peritrophic matrix and the midgut epithelium of the mosquito invade and exits several epithelial cells before emerging on the basal side of the epithelium to develop into an oöcysts (Good, 2001). The invasion process is similar to other Apicomplexa, except that the oökinete neither have rhoptries nor form a parasitophorous vacuole after invading the host cell. The oöcysts undergo an asexual replication, called sporogony, which culminates in the production of several thousand sporozoites, which generally takes 10-28 days depending on the species and temperature. Upon maturation the oöcyst ruptures and releases the sporozoites which cross the basal lamina into the haemocoel (body cavity) of the mosquito.

These sporozoites are motile and have an ability to specifically recognize the salivary glands. After recognition, the sporozoites invade and transverse the salivary gland epithelial cells and positions within its lumen. Some of these sporozoites are expelled into the vertebrate host as the mosquito takes a blood meal, and thus reinitiate the infection in the vertebrate host (Baton and Ranford-Cartwright, 2005). *P. vivax* sporozoites invade the salivary glands more than two days earlier than *P. falciparum* sporozoites, i.e. ≥ 11 days after the infected bite for *P. vivax* versus ≥ 13 days for *P. falciparum* (Reviewed in Sattsbongkot *et al.* 2004).

1.6. Host cell invasion by the malaria parasite

Unlike many other pathogenic organisms, *Plasmodium* species can efficiently infect their hosts by rapidly gaining entry to cells using their own invasion apparatus to identify, penetrate, and establish themselves. Host cell entry by *Plasmodium* represents an obligatory and parasite-specific process (Miller *et al.* 2002a).

The sequence of events for invasion is probably similar for all *Plasmodium* species; the parasite must engage in binding receptors on the RBC, and undergo apical reorientation, junction formation, and signalling (Weatherall & Clegg, 2002). To invade the host cell, the parasite must first initiate a path out from its infected cell, and this process involves disruption of the parasitophorous vacuole, and the host-cell. Three organelles on the invasive (apical) end of the parasite (rhoptries, micronemes and dense granules) define the phylum Apicomplexa. Receptors that mediate invasion of RBCs by merozoites and invasion of liver by sporozoites are found in micronemes on the cell surface and in rhoptries. The location of these receptors within organelles may protect the parasite from antibody-mediated neutralization. Furthermore, proteases are involved which occur in a two-step process such that destruction and rupture of the internal vacuolar membrane occur distinctly prior to that of the erythrocyte membrane (Salmon *et al.* 2001; Wickham *et al.* 2003). Candidate proteases include members of the serine repeat antigen (SERA) family that is localized in the parasitophorous vacuole (Miller *et al.* 2002b; Hodder *et al.* 2003). Subsequent to rupturing the hepatic cells, merozoites recognize, attach, and enter erythrocytes rapidly, probably within 60 seconds. The short time frame is highly efficient for the parasite as antigens on the surface of the extracellular form of the parasite are vulnerable to immune attack (Cowman & Brendan, 2006).

To enter the cell, a so-called tight junction is formed between the parasite and host membrane. The tight junction moves from the apical to the posterior end of the merozoite in a complex series of events powered by the parasite actin-myosin motor (Keeley & Soldati, 2004). One of the most conspicuous events is the removal or "shedding" of the fuzzy coating covering the merozoite surface, a proteolytic event involving SUB2, a serine protease localized in the apical microneme organelles (Harris *et al.* 2005a). As the tight junction moves toward the posterior end, the ligands mediating the invasion process must be removed. By analogy with *Taxoplasma gondii*, it appears likely that, in *P. falciparum*, this may involve the rhomboid family of serine proteases which, unusually clip proteins from within the phospholipid bilayer (Brossier *et al.* 2005; Dowse *et al.* 2005). As the parasite pushes its way into its host cell, it creates a parasitophorous vacuole to seal itself from the host-cell cytoplasm and form an environment hospitable for its development.

Malaria parasites have intracellular signalling pathways mediated by phosphoinositide, cyclic adenosine monophosphate (cAMP) and calcium-dependent mechanisms. Invasion events include releasing essential molecules from apical organelles and initiating the actin–myosin

moving junction that brings the parasite inside the vacuole that forms in the RBC. The thrombospondin-related adhesive protein (TRAP) protein interacts with skeletal proteins in malaria sporozoites and in *T. gondii* (Silvie *et al.* 2004), but the equivalent molecule for merozoites has yet to be identified. Which merozoite surface molecules recognize the RBC surface and signal the start of the invasion process is completely unknown.

1.7. The pathogenic and epidemiological nature of *P. vivax* malaria

Several biological characteristics underlie the distinct pathogenic and epidemiological nature of vivax malaria. *P. vivax* has its apparent preference for invading young red cells (reticulocytes), causing severe anaemia by dyserythropoiesis and destruction of infected and uninfected erythrocytes (recurrent haemolysis) despite much lower parasitaemias (Collins *et al.* 2003; Carlton *et al.* 2008). In certain areas these attributes of vivax malaria are likely to be the most important determinants visible from young infants and children (Price *et al.* 2007). The selective preference for red cell invasion is an imperative factor limiting the growth of *P. vivax* both *in vitro* and *in vivo*. *P. vivax* is capable of inducing fever at levels of parasitaemia lower than those causing fever in *P. falciparum* infection, thus, the host inflammatory response is activated to a greater extent during infections with *P. vivax*, with plasma levels of fever-inducing cytokines such as tumour-necrosis factor-α (TNF-α) being higher in vivax malaria compared with *P. falciparum* infections with similar parasitaemia (Karunaweera *et al.* 1992; Hemmer *et al.* 2006).

P. vivax has a longer incubation period (12 days to several months), a similar erythrocytic cycle (42-48 hours), and produces fewer merozoites per schizont and the merozoites require a single cell receptor, the Duffy antigen (a trait shared with the closely related monkey malaria parasite *P. knowlesi*), to invade host erythrocytes. Humans lacking the antigen are not susceptible to infection, explaining why *P. vivax* is largely absent from West Africa, a highly malarious region where the Duffy negative blood group is ubiquitous (Reviewed in Carlton *et al.* 2008). While, this paradigm was challenged by observations in East Africa, demonstrating *P. vivax* transmission in a population found to be Duffy antigen negative (Ryan *et al.* 2006), limitations of *P. vivax* invasion led to the discovery of two families of parasite receptors; the parasite molecule that binds to the Duffy blood group system with homologous Duffy-binding-like (DBL) proteins of *P. falciparum* and *P. knowlesi* and second, the parasite reticulocyte-binding proteins of *P. vivax* with homologous reticulocyte-binding-like (RBL) proteins of *P. falciparum* and *P. yoelii*. Various members of the DBL and RBL families may

recognize different RBC receptors to those of the Duffy blood group or the receptor on reticulocytes (Reviewed in Miller *et al.* 2002a).

Dynamic differences in *Anopheles* mosquitoes allow *P. vivax* transmission in temperate climates which is not tolerated by *P. falciparum*. In such regions *P. vivax* infects hepatocytes but may persist as dormant hypnozoites for months or years before initiating blood stage infections (relapses) during another transmission season. *P. vivax* strains from different geographic areas display widely different relapse patterns, reflecting evolutionary adaptation to local environmental conditions that optimize transmission potential of the parasite. Strains from tropical areas are characterized by early primary infection followed by frequent (10–14) relapses 3–6 weeks apart (Reviewed in Price *et al.* 2007), whereas in temperate areas primary infection tends to occur later with intervals up to one year and fewer and later relapses to overcome prolonged periods of the year when transmission to the mosquito vector is unfavourable (Baird and Hoffman, 2004). The risk of relapse with tropical *P. vivax* often exceeds 50% within a month of the primary attack, and multiple relapses are the rule, while among strains from temperate regions, the risk of relapse typically ranges from 5 to 25%, and multiple relapses are rare (Baird, 2004). The ability to relapse renders *P. vivax* resilient to eradication from both the human host and the environment.

1.8. Prevention and control

Early diagnosis and effective treatment can cure infection, prevent further morbidity and progression to severe disease and death, and arrest transmission (Breman *et al.* 2006). This intervention requires timely and accurate diagnosis; such as use of efficacious drugs, education of patients and their families about the disease, home management and referral to higher levels of the health system. Effective curative treatment (finding the most suitable alternative to Chloroquine), and better relapse prevention (long-acting drugs such as Dihydroartemisinin, Piperaquine) decrease early relapse compared to other artemisinin-based combination therapies in *P. vivax* (Ratcliff *et al.* 2007). The combination of CQ for blood stages and PQ for liver stages remains the current drug regimen for vivax malaria (Reviewed in Sattabongkot *et al.* 2004) and chemoprophylaxis is advised by travel medicine specialists for non-residents of endemic areas who are exposed to malaria for short periods, though WHO does not recommend chemoprophylaxis for permanent residents of endemic areas because of low feasibility and compliance and high costs relative to the public health benefits (WHO 2000a, 2000b). The choice of chemoprophylaxis will depend on the drug-sensitivity

profile, tolerance, side effects, costs, regimen, and compliance by patients and the effectiveness of chemoprophylaxis will depend mainly on patient compliance and parasite susceptibility (Kain *et al.* 2001; Baird, 2005; White & Breman, 2005).

In areas of high malaria transmission and poor access to facility-based health care, particularly in rural Africa, roll back malaria (RBM) advocates home management of children less than 5 years of age with malaria as a strategy to achieve high coverage of prompt and effective antimalarial treatment in this highly vulnerable group (WHO/HTM/MAL/2004.1096). This involves educating mothers, training community-level providers (including shopkeepers) and supplying pre-packaged quality assured medicines.

Predicting the emergence and spread of resistance to current antimalarials and newly introduced compounds is necessary for planning malaria control and instituting strategies that might delay the emergence of resistance (Hastings & D'Alessandro, 2000). Control of malaria requires case detection and treatment of affected individuals and for curtailment of malaria transmission, control of mosquito vectors is undertaken. Vector control requires knowledge of the ecology of breeding and resting habitats and behaviour of various species of mosquitoes (Dhiman, 2000) and insecticide-treated nets (ITN), indoor residual spraying (IRS) for selected areas, epidemic preparedness and surveillance are key control strategies in all malaria affected countries (World Malaria Report, 2005).

The use of bed nets, curtains, and other materials to provide personal protection by killing or repelling mosquitoes is one of the major strategies of malaria control (Roll Back Malaria, 2002). Pyrethroids are recommended for the periodic treatment or retreatment of the protective materials. The effectiveness of ITNs depends on their acceptability by the population at risk and their affordability, contingent on the habits, biology and susceptibility of the mosquito vector, the compliance of the human population, and the concentration of insecticide on or in the fiber, which has to be maintained by regular re-treatment or by incorporating the insecticide in the fiber for an extended period of time. Over 20 studies in Africa and Asia demonstrated more than 50 percent protective efficacy for individual users of ITNs in reducing malaria episodes, 29 percent protection against severe malarial disease, and substantial protection against anaemia. Importantly, the use of ITNs reduced child mortality by 18 percent in five sites in Sub-Saharan Africa and demonstrated the efficacy of ITNs in both stable and unstable transmission areas (Lengeler, 2004). Re-treating ITNs semi-annually or just prior to the annual peak in transmission can effectively control the vector but harbours

a major logistical and financial challenge. Fortunately, new types of nets with long lasting insecticidal property are now available, and retreatment will soon cease to be an issue (Breman *et al.* 2006).

The reduction of *Anopheles* breeding and biting of humans involves different methods of insecticide and repellent application, environmental management, and behavioural change of populations at risk. Indoor residual spraying (IRS) is the application of long-lasting insecticides (up to six months) on the walls of dwellings. Insecticides repellents obstruct mosquitoes from entering houses or impart a lethal dose of the insecticide on the female mosquito when it rests on a sprayed surface. IRS is most effective against indoor-biting (anthropophilic and endophilic) mosquito vectors (Sattabongkot *et al.* 2004). Vector susceptibility and post feeding behaviour are the main criteria to be considered when choosing an insecticide. Organophosphates, Carbamates, and Pyrethroids are the main compounds used, although some countries still rely on Organochlorines (dichlorodiphenyltrichloroethane, or DDT). The effectiveness of this intervention depends on cost, toxicity, acceptability of the insecticide and its residual effects. Malaria incidence decreased sharply following the use of IRS in large scale programs in many parts of Africa, the Americas, Asia, and Europe (Lengeler & Sharp, 2003). Control was initiated through the use of IRS and supported by engineering approaches, larviciding, prompt diagnosis, and effective treatment.

Larviciding is the application of chemical insecticides, including those of biological origin and insect growth regulators, to breeding sites (Breman *et al.* 2006). These may be applied to all mosquito breeding sites or targeted on the breeding sites of specific vectors. Recommended compounds, formulations, and dosages for larviciding are available through the WHO Pesticides Evaluation Scheme. Fogging or space spraying with insecticides requires specialized equipment, as the particle size of the insecticide determines its suspension qualities in the air, the number of droplets, and the penetration of space. Despite its impact on mosquito density and its contribution to reducing transmission, larviciding is not as effective as IRS and ITNs in reducing mosquito longevity (Najera & Zaim, 2002), and the application must occur during periods of peak target mosquito activity, generally at night. Larviciding is not generally indicated for large-scale vector control in rural endemic areas due to the difficulty of locating all breeding sites, thus, larviciding is usually limited to urban areas, refugee camps, and industrial and development projects. Draining and filling larval breeding sites is one of the oldest methods of mosquito control and must be targeted to the breeding

sites of locally important malaria vectors. Civil engineering strategies that require costly heavy equipment and materials are useful for eliminating permanent breeding sites in urban areas, which are increasing globally.

Commercially available mosquito repellents are applied directly on the skin or clothing as aerosols, lotions, or creams and contain active ingredients that protect the individual from mosquito bites. Some communities in endemic regions use smoke, burning herbs, or plants to deter mosquitoes from entering the home. Commercially available mosquito coils containing Pyrethroids can be burnt to repel mosquitoes, and electrically heated dispensers serve a similar function. N, N-diethyl-meta-toluamide (DEET) is the most widely used and effective ingredient in commercially available repellents. While several studies have shown that mosquito coils are effective at repelling mosquitoes, they are not as effective as ITNs (Reviewed in Breman *et al.* 2006).

The difficulty in controlling *P. vivax* has been exacerbated for several reasons. First, the developmental biology of *P. vivax* is unique in early gametocytogenesis and the generation of hypnozoites in the liver that are responsible for relapses of the disease. Second, the responses of *P. vivax* to some antimalarials differ when compared to *P. falciparum*. Third, the behaviour and physiology of mosquito vectors, especially cryptic species, are largely unknown in many regions where *P. vivax* is endemic (Sattabongkot *et al.* 2004). The successful control of *P. vivax* transmission in larger malaria-endemic areas will depend on a precise definition of local epidemiology and consistent government support. Four important steps to control malaria will be; i) early diagnosis and prompt treatment, ii) promoting health education to populations at risk, iii) vector control by indoor residual spraying and ITNs and iv) monitoring insecticide resistance (Sattabongkot *et al.* 2004).

1.9. Drug and insecticide resistance

Emergence and spread of drug-resistant parasites poses a major difficultly for the management of malaria in endemic areas (Uhlemann *et al.* 2005). Southeast Asia has the highest rates of drug and insecticide resistance in the world (World Malaria Report, 2005), while resistance is the most likely explanation for a doubling of malaria attributable child mortality in eastern and southern Africa (Korenromp *et al.* 2003).

Chloroquine (CQ) still remains the first line of treatment for patients with vivax malaria, as it is well tolerated and affordable, and its long half-life provides protection from early relapses following treatment (Rajakaruna *et al.* 2008). The first cases of Chloroquine resistant *P. vivax* malaria were reported in 1989 from Papua New Guinea (PNG), northern Papua New Guinea (formerly Irian Jaya) and Indonesia and Chloroquine monotherapy is now virtually ineffective in PNG and Indonesia with significant clinical resistance apparent throughout the Indonesian archipelago. Moreover, sporadic cases have been reported from Myanmar, South America, Vietnam and Turkey in recent time (Reviewed in Suwanarusk *et al.* 2007). Drug efficacy tests are often confounded by the relapse of vivax malaria. Occasionally relapses are identified by the length of time between initial and subsequent infections, where the relapse interval of Southeast Asian *P. vivax* strains coincides with the mean time when recrudescence occur following drug treatment (Reviewed in Sattabongkot *et al.* 2004). However, in certain cases recrudescence from a relapse is undistinguished when using available genotyping methods as the strains causing relapse and recrudescence might have derived from the same initial infection.

Currently, mechanisms of *P. vivax* resistance to CQ are ambiguous but appear to differ from *P. falciparum*. Nonetheless, Baird (2004) demonstrated genetic determinants resistance to CQ in *P. falciparum* with mutations in the chloroquine resistance transporter (*crt)* gene, mutations incriminated in *P. falciparum crt* were lacking among CQ-resistant *P. vivax* isolates, and no other mutations in the gene correlated with the phenotype. Using a carefully defined sample set, Suwanarusk (2007) tested the associations between *in vitro* response to Chloroquine and polymorphisms of the orthologues of *P. vivax mdr* like gene (*Pvmdr-1*) and *P. vivax* chloroquine resistance transporter (*Pvcrt-o*) genes. Though, sequence polymorphism was observed in both genes, a lack of association with increased CQ resistance was apparent, but, the P*vmdr-1* polymorphism may provide a useful tool to highlight areas of emerging CQ resistance. Though, novel genes are involved in CQ resistance in *P. vivax*, identification might be complicated due to the difficulties involved in performing genetic crossing and quantitative trait loci mapping of the resistant gene(s), for such studies, animal models of malaria are highly advantageous (Carlton *et al.* 2001).

Apart from differences in molecular mechanisms, slower development of CQ resistance in *P. vivax* than in *P. falciparum* is plausible due to differences in the biology of gametocytogenesis between the two parasite species. For example, *P. falciparum* gametocytes form after the appearance of symptoms and drug treatment, and hence favour the transmission

of resistant genotypes (Sutherland *et al.* 2002), while, early *P. vivax* gametocytogenesis allows the parasite to transmit before the symptomatic stage of the disease and anti-blood stage chemotherapy occur.

Sulphadoxine (SP) was considered an ineffective drug against *P. vivax* malaria due to the immediate appearance of resistance in the parasites, which led to believe that *P. vivax* was intrinsically resistant to SP (Reviewed in Sattabongkot *et al.* 2004). A more plausible explanation was that heavy deployment of the antifolates against *P. falciparum* infection had selected resistant *P. vivax* strains. Indeed, the mechanism of resistance to SP in the two *Plasmodium* species appears to be identical (Imwong *et al.* 2003). Importantly, innate resistance to Sulphadoxine has already been described in *P. vivax* (Sharrock *et al.* 2008). Innate resistance to the antifolate Sulphadoxine was due to a substitution on the *pvdhps* SP binding site conferring a significantly lower sensitivity to Sulphadoxine in *P. vivax* (Auliff *et al.* 2006). However, the ring stage and trophozoites of *P. vivax* are equally insensitive to Sulphadoxine, unlike the stage specific effect of Chloroquine on the ring stages of *P. vivax* (Sharrock *et al.* 2008). In Ethiopia, SP was replaced by Artemether/ Lumefantrine (AL) five years later after a study conducted in 2004, however treatment failure was recorded in 11 sites (Teka *et al.* 2008).

Pyrethroids are the only class of insecticides that are recommended by the WHO and the Centers for Disease Control and Prevention for net impregnation to control malaria transmission ([4]CDC, 2007). Unfortunately, malaria vector resistance to Pyrethroids is becoming widespread across malaria endemic regions (N'Guessan *et al.* 2007; Pennetier *et al.* 2008; Perera *et al.* 2008). At present, Fenitrothion (an organophosphate), Pyrethroids and Deltamethrin are the major insecticides used for IRS in Sri Lanka (Perera *et al.* 2008). Resistance may develop due to changes in the mosquito's enzyme systems, resulting in more rapid detoxification or sequestration of the insecticide, or due to mutations in the target site preventing the insecticide-target site interaction (Hemingway *et al.* 2004). Insecticides that can be used in malaria control are increasingly becoming limited, thus, the only option for managing insecticide resistance in malaria vectors is, the optimal use of existing compounds. Two such tactics are; the alternating use of different classes of insecticides by rotation of active ingredients and mosaic treatments (Guillet *et al.* 2001; Hougard *et al.* 2003), and the use of insecticide mixtures (Hougard *et al.* 2003; Asidi *et al.* 2005). Where IRS is employed, bioassay assessment of the sensitivity of *Anopheles* to the insecticides used must be done periodically using WHO protocols together with toxicity monitoring of spray personnel

(WHO, 2003). Long-duration treated nets should retain insecticide potency, and the materials should be inspected and assayed for confirmation of quality and amount of the chemical (Smith *et al.* 2007); nets that require dipping every 6 months require chemical analysis periodically to assure potency. Sham or counterfeit drugs are now a major problem in Asia and Africa, and antimalarials are a target for the counterfeiters (Breman and Holloway, 2007). A plan needs to be developed to survey the drugs, nets, insecticides, and other supplies to assure they meet national and international specifications.

1.10. Immunity to malaria

Asexual blood stages of the *Plasmodium* life cycle are responsible for the acute symptoms of malaria. High fever and rigors are more common in *vivax* than *falciparum* malaria, reflecting synchronicity of schizont rupture. The classic paroxysms of fever lasting 4–8 hours and occurring with 48–56 hour periodicity, take several asexual cycles to develop in primary infections, although relapses often proceed synchronously with rigors (Collins *et al.* 2004). Differences in parasite virulence, host susceptibility, age of exposure and parasite factors such as antimalarial drug resistance may play a crucial role in modulating the clinical presentation. Specifically, immunity to asexual blood stages in the infected host is important in reducing the clinical burden and elimination of the parasite load (Casey *et al.* 2004). Innate factors influencing malaria are those specific characteristics of a host that are present from birth. For example, individuals carrying the sickle cell trait (heterozygote's for the abnormal haemoglobin gene) will be relatively protected against severe disease and death caused by *P. falciparum* malaria (Reviewed in Weatherall & Clegg, 2002).

Immunity against malaria can be of different categories; one which reduces the risk of death from malaria and another which reduces the intensity of clinical symptoms (Carter and Mendis, 2002). A third type of protective antimalarial immunity is antiparasitic immunity, which directly reduces the numbers of parasites in an infected individual. These are epidemiological definitions of immunity, while the actual cellular and molecular mechanisms of immunity may overlap to a considerable degree between these categories. The numbers of malarial inoculations experienced and the intervals between them, are all important for the malaria immune status of an individual (Reviewed in Kiszewski & Teklehaimanot, 2004). However, clinical immunity to other non life threatening clinical effects of malaria requires more and frequent inoculations of malaria. Effective anti-parasitic immunity is achieved only after many and frequent infections.

Acquired immunity to malaria are evident in two forms; i) immunity which can protect against the parasite ever establishing in a human host, *i.e.* developed immunity against the sporozoites and the early non-pathogenic stages in the liver following long exposure to intense malaria transmission, and ii) immunity against malaria parasites in the sexual blood stages of the parasites which are infective to mosquitoes. Antibodies against antigens of gametocytes and gametes of *Plasmodium* develop readily during the course of natural infections and may affect the transmission dynamics of malaria in certain endemic situations. Anti-sexual stage immunity, however, has no direct relevance to the status of an individual with respect to an individual's experience of a current malarial infection (Carter & Mendis, 2002), thus, immune mechanisms activated by the infection are able to block parasite transmission from the patient to the mosquito vector via transmission blocking immunity (Tsuboi *et al.* 2003). This immunity may play an important role in decreasing the spread of malaria, particularly in areas of low transmission intensity. Sera from individuals naturally exposed to *P. vivax* and having acute infections contain antibodies to gametocytes capable of blocking the parasite fertilization process and the oökinete invasion of the mosquito midgut cells, thus preventing further parasite development (Arévalo-Herrera *et al.* 2005b). However, the process of blocking is complex and appears to be dependent on antibody concentration. Approximately half of the serum samples collected in Kataragama, a *P. vivax* endemic area of Sri Lanka, was analyzed for their transmission blocking activity to suppress gametocyte infectivity, and 12% demonstrated enhancement of malarial transmission (Gamage-Mendis *et al.* 1992).

Individuals in malaria endemic areas frequently have premunition, *i.e.* parasitaemia and antibodies (Ab) without symptoms. Acquired protective immunity induced by malaria parasites involves both Ab-mediated and cell-mediated immunity (Wipasa *et al.* 2002). It is entrenched that B cells and Ab play a crucial role in immunity to malaria. Naturally acquired immunity against malaria in individuals living in endemic areas takes several years to develop and depends largely on the acquisition of a repertoire of specific protective Abs directed against the polymorphic target antigens of the asexual erythrocytic stage of the parasites life cycle (King *et al.* 2008). The degree of protective immunity in humans (Wickramarchchi *et al.* 2006) monkeys (Arevalo-Herrera *et al.* 2005) and mice (Yazdani *et al.* 2004) were shown to correlate with the level of Ab against asexual blood stage antigens, and are dependent on Ab isotypes. Cytophilic Ab of the IgG1 and IgG3 subclasses are considered to be the most important Abs for protection against *P. vivax* malaria in humans (Wickramarchchi *et al.* 2006), acting in collaboration with effector cells such as monocytes and macrophages to

mediate opsonisation and Ab-dependent cellular inhibition. IgG2 may be involved in protection (Aucan *et al.* 2000) and IgG4 which competes with cytophilic Ab for antigen recognition may block cytotoxicity mediated by Ab activated effector cells (Wipasa *et al.* 2002).

Antibody responses directed against surface proteins on the merozoite may function either by blocking RBC invasion or by making the merozoite susceptible to phagocytosis. Antibodies to molecules on the merozoite surface break the blood cycle by blocking merozoite invasion of new RBCs. Antibodies to the glycosylphosphatidylinositol (GPI) anchored proteins, one of the putative immune stimulatory molecules, could suppress the activation of macrophages, resulting in failure of production of pro-inflammatory cytokines (Schofield *et al.* 2002). Parasite antigen-specific Abs play an important role in controlling parasitaemia via Ab-dependent cellular inhibition (ADCI), where binding of antibodies to phagocytes through Fc receptors leads to inhibition of parasite growth (Aucan *et al.* 2000; Tebo *et al.* 2001). Specific Ab initiate parasite clearance by opsonisation, thus enhancing the activity of phagocytic cells or initiating complement mediated damage (Giribaldi *et al.* 2001). Serum Ab from malaria immune donors can also bind to surface accessible regions of the merozoite forming an immune complex, leading to agglutination and inhibition of merozoite dispersal (Nebie, *et al.* 2008).

Antibodies to malaria antigens of *P. falciparum* prevent cytoadherence and may thus prevent development of cerebral malaria (Michon *et al.* 2000; Smith *et al.* 2000). Moreover, blocking cytoadherence could also prevent infected RBCs from sequestered in the periphery, thus presumably allowing them to be removed by the spleen. Antibodies can disrupt spontaneous binding of uninfected RBCs to *Plasmodium*-infected RBCs (rosetting), which also associates with cerebral malaria. Despite the importance of Ab responses for protection against malaria, not all Abs are protective. Monoclonal antibodies (mAb) against MSP-1_{19} which inhibit RBC invasion by merozoites and prevent MSP-1 secondary processing, can be blocked by other mAb to the same antigen (Patino *et al.* 1997). These studies illustrate the importance of identifying epitopes that induce protective Ab when designing a vaccine against malaria.

The overall protective immunity in malaria, apart from antibodies is mediated by a combination of T cells, cytokines, and several other mechanisms that act at different levels of the parasite life cycle. Some of these mechanisms appear to be more relevant for blocking of certain parasitic stages (Hisaeda *et al.* 2005) and it was evident that T cells play crucial roles

in protective immunity against malaria parasites (Reviewed in Hisaeda *et al.* 2005). Cluster determinant factor 8 (CD8+) T cells exhibit cytotoxicity to hepatocytes, where the release of merozoites from the liver into the bloodstream is prevented and the infection is terminated before disease onset. CD4+ T cells are indispensable for protection against blood stage parasites by providing assistance for antibody production and by secreting Interferon γ (IFNγ) for activation of macrophages to promote clearance of infected RBCs (Hisaeda *et al.* 2005).

Maitland and colleagues (1997) suggested that the earlier age peak for *P. vivax* disease may protect against the later acquired *P. falciparum* through species transcending immunity. Studies have shown that symptoms of *P. vivax* and *P. falciparum* infections were significantly reduced by recent previous *P. vivax* infections (but not for *P. falciparum* infection) (Gunawardena *et al.* 1994). If previous vivax infections does protect against severe *P. falciparum* infections, further investigations are needed so that policies for *P. vivax* control can balance reduction in severe vivax with possible loss of vivax-associated protection against severe *P. falciparum* infection (Rogerson & Carter, 2008).

However, to acquire long lasting immunity is difficult despite frequent exposure to parasites in endemic regions. Many reasons are presented for the low immunogenicity of malaria parasites. Hisaeda *et al.* (2004) revealed that CD4+, CD25+ regulatory T cells play a crucial role in interfering with the initiation of protective immunity in very early stages of the infection in a rodent model. However, many issues primarily including the complicated and inscrutable evasion strategies of the parasite should be elucidated before the establishment of an effective method for controlling malaria, including the development of effective vaccines. Several immune evasion mechanisms of malaria parasites include, intracellular parasitism, adherence of infected red blood cells to endothelial cells, antigenic diversity / polymorphism and molecular smokescreen (Hisaeda *et al.* 2005).

Intracellular parasitism is a primitive escape mechanism of microbes, where antibodies bind to free sporozoites or merozoites, but the process is hindered once the parasites enter the host cells. Furthermore, merozoites escape recognition by CD8+ T cells when RBCs lack major histocompatible complex (MHC) molecules on their surface (Hisaeda *et al.* 2005).

Adherence of infected RBCs to endothelial cells is responsible for the severe pathogenesis of the disease. This mechanism might have been originally acquired as an immune evasion

strategy, since adherence may protect the parasites from entering the spleen and liver, where mature parasites are trapped and digested (Hisaeda *et al.* 2005).

Antibodies and T cells recognize antigen epitopes in a very specific manner. Therefore, changing antigenicity is a major strategy for parasites to escape from host immunity. One strategy is antigenic diversity/polymorphism, which involves the expression of antigenically different alleles of a gene in different parasite populations and presumably results from selection under immune pressure. In the presence of specific immunity, parasites expressing mutated alleles that are not recognized by the immune system would selectively expand. In fact, antigens eliciting immune responses often show extensive polymorphisms, which import a troublesome hurdle to overcome in the development of effective malaria vaccines. Merozoite Surface Protein-1 (MSP-1), a leading vaccine candidate, has many alleles (Thakur *et al.* 2008a), while antibodies to one allele of the Apical Membrane Antigen-1 (AMA-1) lacked recognition to the other alleles (Cortés *et al.* 2005). Antigen polymorphism affects T cell recognition more severely, as T cell recognition depends on the primary structure (amino acid sequence) of proteins rather than protein conformation, which is often recognized by antibodies. Substitution of only one amino acid in a protective T cell epitope results in the failure to activate protective T cells (Putaporntip *et al.* 2002).

Tandem repeats are found in many malaria antigens, which provide immune dominant B cell epitopes. Individuals who have had repeated infections have high concentrations of antibodies to these regions in serum, though they are still susceptible to new infections, suggesting that antibodies that contribute to a lesser extent to protection are produced. Together with the generation of antigen diversity, it is postulated that tandem repeats have a pivotal function in immune evasion. The presence of polymorphic repeats in antigens that are not targets of protective immunity affects affinity maturation of antibodies and thus masks the critical epitopes. Tandem repeats also induce T cell-independent B cell activation by cross-linking their surface immunoglobulins and suppressing antibody responses to important adjacent regions (Hisaeda *et al.* 2005).

1.11. 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 (Breman *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).

Development of vaccines for malaria is more 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 & Saul, 2002). However, there is accumulating evidence to suggest that anti-malarial vaccines can be developed successfully. Historically, since the mid 1980s limited focus on *P. vivax* vaccine development was apparent, and this is reflected in the malaria vaccine 'rainbow tables', with only a few *vivax* vaccine candidates listed among 56 preclinical programmes and 20 clinical studies (4http://www.who.int/vaccine_research/documents/en). A few laboratories have worked to identify and pre-clinically characterize *P. vivax* vaccine candidates, with only a couple of *P. vivax* antigens imminently poised to advance into clinical trials (Galinski & Barnwell, 2008).

Different stages of the parasite express different repertoire of antigens, and many proteins of the parasite exhibit remarkable polymorphisms. Host genetic determinants, for example, specific polymorphisms in immune response genes, may profoundly influence the immunity towards individual antigens (Perlmann & Troye-Blomberg, 2002). A vaccine combined with other control strategies, would be a sustainable way to combat malaria (Nussenzweig & Long, 1994) while the brunt of malaria related morbidity and mortality is borne by children less than five years of age and pregnant women, a subunit or recombinant based vaccine should be targeted to these groups. Testing malaria vaccines in young children may be best accomplished in a population in which at least four parameters are known: the malaria related morbidity and mortality, malaria transmission pressure, the molecular nature of the infectious agent, and the characteristics of naturally acquired immune responses that confer protection against parasitaemia and clinical illness.

Three principal targets for a malaria vaccine would be – i) the pre-erythrocytic stage (sporozoite and hepatic stages), ii) the asexual erythrocytic stage and iii) the sexual or transmission stages (Reviewed in Phillips, 2001).

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4 WHO Vaccine Development Rainbow Tables {http://www.who.int/vaccine_research/documents/en}

1.11.1. Pre-erythrocytic (sporozoite and liver stage) vaccines

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. Although sterile immunity has been achieved by vaccination of experimental animals and humans using irradiated sporozoites, the mechanisms of protective immunity are poorly understood (Reviewed in Herrera *et al.* 2007). Currently, cytokines that inhibit parasite development and antibodies are thought to block sporozoite invasion contributing to protection, while Interleukin 12 (IL12), produced by macrophages, dendritic cells and kupffer cells, induces CD8+ T cells and natural killer cells to produce interferon-gamma (IFN- γ), which activates hepatocytes to produce reactive metabolic intermediates (e.g. nitric oxide) that are toxic to intrahepatic parasites (Artavanis-Tsakonas *et al.* 2003; Riley *et al.* 2006). Although multiple *P. falciparum* pre-erythrocytic vaccine candidates are being tested, only two *P. vivax* sporozoite antigens, the Circumsporozoite protein (CSP) and the Sporozoite Surface Protein2 / Thrombospondin Related Adhesive Protein (SSP2/TRAP) are identified so far (Herrera *et al.* 2007).

Unlike *P. falciparum*, *P. vivax* has two different alleles of the *csp* gene VK210 and VK247 or type I and type II, respectively, with the encoded repeat amino acid unit differing between the two alleles (Reviewed in Galinski & Barnwell, 2008). Currently, *Pvcsp* vaccine candidates are being made in the form of chimeric recombinant proteins expressed in *E. coli* and encoding both types of repeat units (Yadava *et al.* 2007), and as three long synthetic peptides corresponding to the N-terminal, C-terminal and repeat regions of *Pvcsp* (Salvador I strain) (Herrera *et al.* 2004 and 2005). *Escherichia coli* expressed chimeric recombinant protein was tested in non-human primate trials and early phase I trials are being planned. The long synthetic peptide vaccine formulated in Montanide ISA 720 has been through trials in both non-human primates (*Aotus l. griseimembra*) and a phase Ia clinical trial showing immunogenicity by eliciting excellent antibody responses assessed by Enzyme Linked Immunosorbant Assay (ELISA) and Immuno Fluorescent Assay (IFA) and γIFN production, the latter particularly in response to live sporozoite exposure in owl (*Aotus*) monkeys (Herrera *et al.* 2005). However, in each of these studies, humans and nonhuman primates were not reported to be challenged with live *P. vivax* sporozoites.

SSP2/TRAP belonging to the transmembrane protein TRAP/MIC2 family is present in sporozoites and liver-stage parasites and mediates adhesion to host cells and tissue surfaces and directs motility and invasion of these cells (Yuda & Ishino, 2004). *P. vivax* SSP2/TRAP contains a single copy of the CSVTCG cell-adhesion motif, which is highly conserved among all malaria circumsporozoite proteins, and the thrombospondin-like sulfatide-binding domain is used by the sporozoite for hepatocyte invasion. The vaccine potential of *P. vivax* SSP2/TRAP has been tested in mice and monkeys where it induced high levels of specific antibodies (Reviewed in Herrera *et al.* 2007). Nonetheless, a region of PvTRAP synthesized as a long peptide and used in pre-clinical studies was immunized to *A. griseimembra* monkeys, which were subsequently challenged with a wild type isolate of *P. vivax* sporozoites acquired by feeding *Anopheles albimanus* mosquitoes on a patient, the trial showed no protection (Castellanos *et al.* 2007).

The pre-erythrocytic malaria vaccine RTS, S which is the chimeric PfCSP/Hepatitis B surface protein construct given with the AS02A adjuvant protected 30% of children against clinical malaria. To date, two studies (both with partial industry support) add new information about candidate malaria vaccines. Bejon *et al.* (2008) evaluated the efficacy of RTS, S formulated with a more immunogenic adjuvant system, AS01E in a double-blind, randomized trial conducted in Kenya and Tanzania, where the efficacy against all clinical episodes of malaria was 56%. Abdulla *et al.* (2008) assessed the feasibility of integrating RTS, S malaria vaccine into the routine infant immunization schedule. In a phase IIb, single center, double-blind study conducted in Tanzania, 340 infants were randomized to receive three doses of RTS, S (AS02D adjuvant), at 8, 12, and 16 weeks of age. Vaccine efficacy for febrile malaria was 59%.

A pre-erythrocytic vaccine based upon either radiation or genetically attenuated sporozoites of *P. falciparum* is strongly supported as experiments performed prior with radiation attenuated live sporozoites, injected in living feeding mosquitoes, were the first direct proof of principle that a human malaria vaccine could be feasible (Clyde, 1990; Mikolajczak *et al.* 2007; Renia, 2008). Previous studies in individuals challenged with *P. vivax* irradiated sprozoite infected mosquitoes have documented protection apparently lasting for 10.5 months (Clyde, 1990). A *P. vivax* attenuated sporozoite vaccine is certainly being considered, but poses a number of challenges (Galinski & Barnwell, 2008). Importantly, a major challenge would be the limited or non-existent sources for reproducibly acquiring standardized *P. vivax* sporozoites for attenuation. This is worth noting, due to the given lack of a practical continuous culture

system that would provide infective gametocytes or the ethical considerations that surround the ability to infect humans with cloned lines of *P. vivax* or a genetically altered line of parasites in order to feed mosquitoes and produce infected mosquitoes with attenuated sporozoites. Efforts are being made to develop a human challenge system for *P. vivax* to evaluate *vivax* malaria vaccines by feeding upon patients with *vivax* malaria in Colombia and Thailand, but drawbacks are evident even as a challenge system (Galinski & Barnwell, 2008).

Pre-erythrocytic stages of the rodent malaria parasites *berghei* and *yoelii* were attenuated by deletion of pre-erythrocytic stage expressed genes called Up-regulated in Infectious Sporozoites (UISs) that resulted in complete arrest of early liver-stage development after hepatocyte infection (Mueller *et al.* 2005a; Tarun *et al.* 2007). Immunization of mice with these UIS negative gene parasites induced complete, long-lasting protection against infectious sporozoite challenge demonstrating that rodent malaria genetically attenuated parasites (GAPs) are highly effective vaccines (Mueller *et al.* 2005a, 2005b; van Dijk *et al.* 2005; Tarun *et al.* 2007). van Buskirk *et al.* (2009) tested whether *falciparum* could be attenuated at the early liver stage by genetic engineering. The *P. falciparum* genetically attenuated parasites (GAPs) harbour individual deletions or simultaneous deletions of the sporozoite expressed genes P52 and P36. The gene deletions did not affect parasite replication throughout the erythrocytic cycle, gametocyte production, mosquito infections, and sporozoite production rates. However, the deletions caused parasite developmental arrest during hepatocyte infection. The double-gene deletion line exhibited a more severe intrahepatocytic growth defect compared with the single-gene deletion lines. Therefore, GAPs might provide a safe and reproducible platform to develop an efficacious whole-cell malaria vaccine that prevents infection at the pre-erythrocytic stage.

1.11.2. Asexual blood-stage vaccines

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). Although *Plasmodium* parasites initially infect hepatocytes, this pre-erythrocytic stage of malaria infection passes silently without any illness and disease results from unrestricted replication of parasites in the blood. 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 & 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.

P. vivax Merozoite Surface Protein-1 (*msp-1*), like *P. falciparum msp-1*, exhibits allelic dimorphism of the Belem and Salvador I strains together with other forms of diversity (Puterporntip *et al.* 2002; Thakur *et al.* 2008a). *P. vivax* MSP-1_{42} and MSP-1_{19} vaccine candidates produced in the baculovirus expression system was tested in a preclinical immunization trial conducted in *Saimiri boliviensis* (squirrel monkeys), a host susceptible to *P. vivax* infections, using FCA as the adjuvant (Reviewed in Galinski & Barnwell, 2008). Another immunization trial in *Saimiri boliviensis* utilized yeast expressed MSP-1_{19} formulated with alum and the non-ionic block copolymer P1005 (Collins, *et al.* 1999). Future trials should be conducted with *P. vivax* strains that are adapted to grow uniformly in non-splenectomised monkeys to be able to more reliably interpret results as the spleen is of central importance in the malaria erythrocytic stage biology and immunity (Engwerda *et al.* 2005). Kaushal *et al.* (2007) characterized the soluble and refolded 42 kDa fragment of *P. vivax* and *P. cynomolgi* expressed in *Escherichia coli* biochemically and immunologically and demonstrated these two antigens to be highly immunogenic in rhesus monkeys and BALB/c mice. A third *P. vivax* MSP-1 vaccine candidate antigen representing a 359 amino acid N-terminal portion of the Belem strain known as Pv200L produced in *E. coli*, displayed strong immunogenicity in BALB/c mice and in Aotus monkeys (Valderrama-Aguirre *et al.* 2005). Primates immunized with recombinant Pv200L formulated in Montanide ISA 720 showed vigorous antibody responses that recognized parasite antigens in western blot and in IFAs. Immunized animals were partially protected against *P. vivax* challenge, as determined by lower parasitaemias, reduced anaemia and spontaneous parasite clearance. This protein is currently being optimized for further preclinical and clinical evaluation.

The genome project has revealed eleven Merozoite Surface Protein-3 genes of similar character in *P. vivax* distributed in a single locus of the Salvador I (Sal I) strain (Carlton *et al.* 2008). All eleven genes encode putative proteins that share the same structural characteristics of a large alanine-rich central domain of heptad repeats forming coiled coils tertiary structure (Galinski, *et al.* 1999 & 2001). Twelve years ago, immunization of *Saimiri boliviensis*

monkeys with two products of the PvMSP-3 family (PvMSP-3α and PvMSP-3β) expressed in *E. coli* using Freund's Complete Adjuvant (FCA), induced strong immune responses in the form of antibodies as determined by IFA and ELISA (Galinski & Barnwell, 2008). Additionally, MSP-3α/β was shown to produce a modest level of protection by reducing peak parasitaemia by 60–70% and attenuating the course of infection in the spleen-intact squirrel monkeys infected with the Belem strain. Recently, immunization of *Saimiri boliviensis* with *E. coli* expressed recombinant 6His-PvMSP-3α formulated in Montanide ISA-720 did not confer protection (Galinski & Barnwell, 2008). Considering the number of MSP-3 genes potentially expressed in *P. vivax* merozoites and the allelic diversity demonstrated by this antigen family, it may be an insurmountable challenge to develop a vaccine based on MSP-3 for *P. vivax*. Conversely, some data suggest that one of the eleven MSP-3 genes may be over expressed relative to the others and this could be a predominantly expressed family member (Galinski & Barnwell, 2008).

Merozoite Surface Protein-9 (MSP-9) is a potential vaccine candidate including an N-terminal region which is conserved and could inhibit merozoite invasion by the production of polyclonal or monoclonal antibodies (Vargas-Serrato *et al.* 2002). Naturally exposed individuals have shown significant humoral and cellular responses, and in fact, an immunodominant epitope has been identified but further knowledge is needed to determine the capability of the epitope to elicit a protective response against vivax malaria (Lima-Junior *et al.* 2008).

P. vivax merozoites require the Duffy blood group receptor for reticulocyte invasion; hence erythrocytes in individuals who lack this blood group are protected from invasion. The parasite ligand for the Duffy blood group receptor is the Duffy Binding Protein (DBP), a functionally conserved protein that belongs to a family of erythrocyte binding proteins (Singh *et al.* 2005). The region II binding domain of DBP which is expressed and refolded in *E. coli* had endured preclinical testing in rodents and non-human primates (Yazdani *et al.* 2004; Arevalo-Herrera *et al.* 2005; Devi *et al.* 2007; Moreno *et al.* 2008). In an immunogenicity study, the *E. coli* expressed and re-folded PvDBP-RII was immunized to *Macaca mulatta*, rhesus monkeys, which showed strong and sustained antibody responses when formulated with Alum, AS02A or Montanide ISA 720 (Moreno *et al.* 2008). The PvDBP-RII vaccine candidate is now being developed for clinical trials pending a reformulation with a suitable adjuvant and satisfactory re-testing in pre-clinical trials. The DBP RII antigen is known to induce the production of antibodies in a variety of species by blocking adhesion of red blood

cells to DBP *in vitro* (Yazdani *et al.* 2004; Arévalo-Herrera *et al.* 2005a; Moreno *et al.* 2008) and antibodies from immunized small animals and infected humans apparently tend to show inhibition of merozoite invasion *in vitro* in a field assay that produces low invasion rates (Grimberg *et al.* 2007; King *et al.* 2008). Grimberg and colleagues (2007) recently demonstrated that antibodies to recombinant PvDBP generated by vaccination of rabbits or affinity-purified from human serum can effectively inhibit reticulocyte invasion by *P. vivax.*

Another potential blood stage vaccine candidate includes the members of the Reticulocye Binding Protein-Like (RBL) family of proteins. *Plasmodium vivax* preferentially invades reticulocytes presumably by the selective attachment of members of the Reticulocyte Binding Protein (RBP) family, such as PvRBP-1 and PvRBP-2 (Galinski *et al.* 2000). Short-term cultured *P. vivax* schizont-infected RBCs for robust *in vitro* merozoite invasion inhibition assays (Singh *et al.* 2003), demonstrated that polyclonal antibodies raised against certain regions of PvRBP-1 and PvRBP-2 expressed in *E. coli* are capable of modestly inhibiting invasion of the *P. vivax* merozoite by 20–40% (Galinski & Barnwell, 2008). Studies with field isolates have endowed relevant data regarding polymorphism and naturally acquired immunity to these proteins (Rayner *et al.* 2005; Tran *et al.* 2005).

Apical membrane antigen-1 (AMA-1) is a member of a family of molecules expressed on the micronemes and surface of merozoites in most rodent, human and nonhuman primate malaria parasites studied. It is an 83 kDa protein in *P. falciparum* and a 66 kDa protein in all other species and has considerable conservation of primary and predicted secondary amino acid structures (Reviewed in Herrera *et al.* 2007). AMA-1 has a role in maintaining parasite growth during the asexual blood phase and the protein is also expressed in sporozoites and, together with TRAP, seems to be involved in hepatocyte invasion (Silvie *et al.* 2004). While, PvAMA-1 is recognized by antibodies in naturally-exposed individuals (Rodrigues *et al.* 2005; Wickramarachchi *et al.* 2006) and monoclonal antibodies to PF83/AMA-1 blocked merozoite invasion *in vitro*, primate experiments using *Plasmodium knowlesi* AMA-1 and *Plasmodium fragile* AMA-1 indicated protective efficacy (Reviewed in Herrera *et al.* 2007). The gene coding for *P. vivax* AMA-1 has been used to construct a nucleic acid vaccine that proved to be immunogenic in mice (Reviewed in Herrera *et al.* 2007), and a recombinant *P. vivax* AMA-1 protein produced in *Pichia pastoris* has proven to be highly immunogenic in rhesus monkeys (Kocken *et al.* 1999).

1.11.3. Transmission blocking vaccines

The target antigens of transmission blocking vaccines (TBV) are exposed on the sexual stages of the parasite, *i.e.* the gamete, zygote and oökinete, in the gut of the mosquito after a blood meal containing circulating male and female gametocytes. Early studies on transmission blocking immunity (TBI) indicated that the primary mechanism of action was through antibody assisted by complement, and more recent studies indicate that antibody- mediated blocking of oöcyst formation is reliable *in vitro* and correlate with TBI (Miura *et al.* 2007). This is considered an important strategy in the control of malaria in areas of low transmission intensity where it would prevent transmission from new foci of infection in the community and would help to control the escape of parasite mutants from vaccines targeted to other stages of the parasite life cycle (Mendis *et al.* 1987; Gamage-Mendis *et al.* 1992; Herrera *et al.* 2007).

The primary candidate antigens for TBV in *P. vivax* are the same suspects as for *P. falciparum*, which include Pvs230, Pvs48/45, Pvs28 and Pvs25. Pv230 and Pv48/45, but are still in line to be brought into preclinical studies primarily due to problems in efficient and structurally sound recombinant expression, although previous studies using monoclonal antibodies indicated that these antigens are important targets of TBI (Reviewed in Galinski & Barnwell, 2008). Pvs25 is thus far the most advanced candidate for a *P. vivax* TBV having endured extensive pre-clinical testing and early phase I clinical trials. Pvs25 can be produced efficiently as a recombinant in yeast (Miles *et al.* 2002) and pre-clinical testing in mice and non-human primates indicated an antibody generation that would have a 70–100% reduction in both the number of infected mosquitoes and in oöcyst burden in infected mosquitoes using adjuvants such as Montanide ISA 720 and Alum (Saul *et al.* 2007a). TBV development also focused on using DNA vaccines encoding Pvs25 and Pvs28, which at least in mice has produced significant blocking antibody titers (Kongkasuriyachai *et al.* 2004). Importantly, pre-clinical selection of other new sexual stage antigens such as CTRP (Kaneko *et al.* 2006) and WARP (Yuda *et al.* 2001) should be expedited along with research on the expression of Pvs230 and Pvs48/45 to broaden and enhance the transmission blocking effectiveness of these vaccines that will be critical for increasing the chances of controlling and eliminating *P. vivax* malaria.

1.11.4. Multi-species vaccine

In view of the potential problems with co-infections, immunoprophylaxis would represent an important complementary control strategy, the urgency of which would be influenced by the progress of a *P. falciparum* vaccine. Currently >70 *P. falciparum* vaccine formulations are available and 23 of these are undergoing clinical testing (Reed *et al.* 2006). Such progress has made possible the goal of having a highly efficacious *P. falciparum* vaccine by 2025 (5http://www.MalariaVaccineRoadmap.net/forum/forum.asp). By contrast, only two *P. vivax* vaccine candidates have recently been tested in humans and a modest number of vaccine candidates have been tested in pre-clinical trials (6http://www.columbia.edu/itc/hs/medical/pathophys/id/2005/LarussaMalariaTable.pdf). Although the current investment in *P. falciparum* is understandable, delays in identifying and developing *P. vivax* subunits for a malaria vaccine may impede the development of a functional malaria vaccine in most malaria-endemic areas. In almost all malaria-endemic regions outside of Africa, and in some places within Africa, these parasite species co-exist (Herrera *et al.* 2007). Therefore, use of a *P. falciparum* vaccine might lead to unpredictable epidemiological outcomes, potentially including an increase in *P. vivax*. Once a *P. falciparum* vaccine becomes commercially available, one might expect a surge of *P. vivax* in Africa. In other endemic areas, including Asian and Latin American regions where *P. vivax* is highly prevalent (60–70%), a hypothetical *P. falciparum* vaccine with a protective efficacy of 50% would result in a decrease in the overall protection against malaria to <20%. As the biology of *P. vivax* is different from that of *P. falciparum*, it might not be possible to extrapolate vaccine approaches from *P. falciparum* to *P. vivax*. Thus, research on *P. vivax* vaccines is a critical need with the design of methods to test multi-species vaccines (Herrera *et al.* 2007).

1.11.5. Multi-stage vaccines

Due to the complex life cycle and antigenic diversity of the malaria parasite, polyvalent subunit malaria vaccines containing multiple protective domains or epitopes from different antigens may be necessary, and may be constructed as chimeric proteins (Pan *et al.* 2004).

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5 Malaria Vaccine Initiative (2005). Malaria vaccine technology roadmap meeting. Malaria Vaccine Initiative, PATH {http://www.MalariaVaccineRoadmap.net/forum/forum.asp}

6 World Health Organization (2005). Portfolio of candidates malaria vaccines currently in development {http://www.columbia.edu/itc/hs/medical/pathophys/id/2005/LarussaMalariaTable.pdf}

Such constructs may provide approaches to address the issue of parasite variation and to enhance the immunogenicity and protective efficacy of vaccination.

Only a short period of time is available for antibodies to act to neutralize the DBP/ Duffy interaction and if much of this invasion ligand or important epitopes are exposed in the moving junction to the host environment is not known. In this regards it has been suggested that the DBP be combined with another merozoite antigen, such as MSP-1_{19}, but a better choice may be AMA-1 as this combination will concentrate the point of antibody attack at the machinery of invasion by neutralizing simultaneously two invasion ligands with different functions that are critical to parasite survival (Devi *et al.* 2007).

1.11.6. New vaccine research approaches

Sequence and initial annotation of the *P. vivax* genome was one development that greatly facilitated the feeding of the vivax malaria vaccine pipeline (Carlton *et al.* 2008), thus relevant vivax malaria vaccine (and drug) interventions will come to light through this data base, even though the field of vaccine development will face the challenge of post-genomic deciphering of the data available from the *P. vivax* genome (Sal I strain) sequence. It is apparent that, targeting the predominant surface coat proteins of sporozoites and merozoites, which were identified in the wake of molecular technological approaches for vaccine development, may not reliably result in effective malaria vaccines capable of inducing high levels of protection (Galinski & Barnwell, 2008). There will indeed be benefits from defining and understanding immunological correlations of protection, and dedicating more effort to testing new robust adjuvants to achieve protective immune responses. Although this applies to malaria vaccines in general, vivax malaria is of considerable and central importance due to the lack of a practical *in vitro* system of continuous cultivation and a more dependant reliance on the use of human clinical cases and non-human primates for propagating the various developmental forms of *P. vivax* together with the relevant simian malarias and investigating host and parasite interactions (Galinski & Barnwell, 2008).

In the absence of *in vitro* culture, the only available sources of material to directly investigate the genetics, biology, metabolism, immunity or pathology of *P. vivax* are infected humans and new world monkeys, although until recently, chimpanzees were only used to study *P. vivax* liver stages. Many clinical studies primarily limited to endemic regions, were able to utilize parasites collected from patients to study genetics, to develop a transcriptome profile or for

TBI and mosquito infectivity (Bharti *et al.* 2006; Bozdech *et al.* 2008). Nevertheless, many aspects of immunity and pathology are also desirably investigated in the intermediate host of *P. vivax*, complete reliance on this source can present challenges and problematic circumstances, but fortunately, *P. vivax* can infect and adapt to a number of species of new world monkeys.

"Humanized" mice were in trend to study blood-stage *P. falciparum*, with hopeful attempts to include pre-erythrocytic stages in these highly immuno-compromised mice by transplanting human liver tissue (Morosan *et al.* 2006; Moreno *et al.* 2007). Similarly, attempts were made to infect "humanized" mice with the blood stages and sporozoites of *P. vivax*, however, it is questionable if these "models" represent solid experimental tools or are merely expensive *in vivo* test tubes of living tissues and organs that will be narrowly targeted and of limited value, which may not provide credible data. Therefore, a greater investment in *P. vivax* vaccine development is required and should include the search for suitable means to test such vaccines.

1.12. Genetic diversity of *Plasmodium*

The fact that diversity in genetic content of individual organisms bears the signature of evolutionary history of species and genes has long been documented ever since the rediscovery of the Mendelian genetics combined with Darwin's theory of evolution by natural selection. 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).

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). Currently, many genes are used for genotyping *P. vivax* field isolates. Parasite surface antigens (e.g. *msp-1*, *msp-2*, *msp-3*, *msp-4*, and *msp-9*) or Polymerase Chain Reaction (PCR) / Restriction Fragment Length Polymorphism (RFLP) of *Pvmsp-3α* genes are used as genetic markers to study parasite diversity, where they contains hypervariable and conserved regions, with the latter demonstrating size polymorphism (Rayner *et al.* 2002; Thakur *et al.* 2008a). Apical Membrane Antigen-1, a protein essential for erythrocyte invasion, is highly conserved in *Plasmodium* species and similar to *Pfama-1*, *Pvama-1* showed single nucleotide polymorphisms (SNP's) within several geographic regions (Gunasekera *et al.* 2007; Ord *et al.* 2008). Similar to *P. falciparum, P. vivax* Circumsporozoite Surface Protein (*csp*) is an extensively studied antigen and is considered to be a major vaccine candidate. Analysis of *Pvcsp* sequences revealed that parasites have repeats belonging to one or two types of repeat units (VK210 or VK247). Both variants have a worldwide distribution, however studies have shown VK210 genotype to be the best adapted variant in the world (Machado & Póvoa, 2000). *Pvgam-1* gene, named due to its expression in the gametocytes, displayed polymorphic sites near the 3' end in limited number of Sri Lankan isolates (Snewin *et al.* 1995) and its allelic dimorphism was recently reported in Indian isolates (Prajapati *et al.* 2006). Region II of *Pvdbp* containing the binding motifs necessary for the adherence of DBP to Duffy antigen receptor for chemokines (DARC) on the erythrocyte surface includes residues that are conserved and highly polymorphic, thus showing varying diversity geographically (Cole-Tobian & King, 2003; Souza *et al.* 2006). Since the information ~~is~~ available is derived from antigens, geographical differentiation caused by positive natural immune selection rather than reduced gene flow might be the cause of high genetic diversity in these genes (Das *et al.* 2007).

Recombination plays an important role in maintaining diversity of *P. vivax*, as recent use of PCR has demonstrated that many individuals in endemic areas have chronic asymptomatic infections often consisting of multiple parasite clones (Bruce *et al.* 2000). It is expected that sexual out crossing and recombination is strongly associated with the intensity of malaria transmission (Gunasekera *et al.* 2007). *Plasmodium* parasites are haploid for most of their life cycle except for a brief period in the mosquito vector in which sexual recombination occurs as an obligate part of the parasite life cycle. For sexual recombination to occur more than one simultaneously infecting parasite genotype per human host must be present, hence it is possible that interrupted feeding by the *Anopheline* vector and consequent sampling of

multiple hosts might also contribute to the diversity of parasites in a blood meal, however, this contribution is considered negligible (Reviewed in Cole-Tobian *et al.* 2005).

Microsatellites are simple sequence tandem repeats that are generally hypervariable, co-dominant and locus specific, used to map traits and to investigate parasite population genetics, where the degree of allelic diversity was demonstrated by several studies (Gomez *et al.* 2003; Leclerc *et al.* 2004; Imwong *et al.* 2006; Russell *et al.* 2006). Ferreira and colleagues (2007) reported extensive genetic diversity and frequent multiple clone infections coexisting with strong multilocus linkage disequilibrium in *P. vivax* isolates obtained during a cross-sectional and longitudinal survey in rural Amazonia. 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).

1.13. Immunoepidemiology of malaria

Immunoepidemiology investigates the influence of population immunity on epidemiological patterns. Monitoring the immune status of a population at appropriate intervals allows estimation of the social, medical and economic importance of infectious diseases, the planning and evaluation of intervention programs, the quick recognition and investigation of unknown diseases, and the discovery of antigenic variation or resistant genotypes associated with parasite protection from host immunity (Hellriegel, 2001). Patterns that emerge of innate, natural and artificially acquired immune responses in a population facilitates the prediction of the spread of infections, for instance on how immunological differences between individual hosts affect the population biology of the disease agents rather than the incidence of disease itself, and, in genetic terms, how the distribution of different types of immune responses in a host population influences the spread of different serotypes or genotypes (e.g. drug resistance) in its parasite population (Hellriegel, 2001). Linking immunological mechanisms to epidemiological patterns takes into account the interrelationship between individual and population levels, and opens up new perspectives. Population immunity depends on the genetic composition of the host population, its history of infection with this and other pathogens, its welfare, and the availability of chemotherapy or vaccination. It reflects the different kinds of parasite dynamics and immune dynamics within heterogeneous host individuals (Hellriegel, 2001).

Despite a long history of attempts to model malaria epidemiology, and most especially that of lethal human malaria *P. falciparum*, the over-riding conclusion is that a detailed understanding of host-parasite interactions leading to immunity is required. The parasite's high degree of genetic diversity and mechanisms of clonal antigenic variation highlights the complex nature of the human-parasite interaction. However, it is still unknown what governs the duration of an infection and how within-human parasite dynamics relate to malaria epidemiology. Paul and colleagues (2007) demonstrated a path to comprehend the immunoepidemiology of malaria, by immunological characterization of the human population stratified by parasite gametocyte allocation. Immunity to *Plasmodium* develops slowly and requires repeated exposure to the parasite, which thus generates age-structure in the host-parasite interaction. An age-structured degree of immunity would present the parasite with humans of highly variable quality. Evolutionary theories suggest that natural selection will mould adaptive phenotypes that are more precise (less variant) in "high quality" habitats, where lifetime reproductive success is best. Variability in malaria parasite gametocyte density is predicted to be less variable in those age groups who best infect mosquitoes. Thus, the extent to which variation in gametocyte density is a simple parasite phenotype reflecting the complex within-host parasite dynamics (Paul *et al.* 2007).

The clinical, parasitological and haematological status of adults exposed to malaria, and characterization of parasites in these individuals who progressively acquire protective immunity were carried out by Mayor *et al.* (2007). It demonstrated that the immune ability to control the parasite may be better reflected by parasite densities rather than by the prevalence of parasites suggesting that immune mechanisms that are able to control parasite densities result in less severe symptoms.

Bastos *et al.* (2007) analyzed the extent to which *Pvmsp-1* sequence diversity affects the development of antibody responses by comparing the naturally acquired antibody responses against three polymorphic domains and the highly conserved C-terminal domain of PvMSP-1 in a cohort of malaria-exposed rural Amazonians. The specific antibodies at the study baseline were measured in the absence of *P. vivax* infection and during subsequent infections. Sequences of the variable domains of *Pvmsp-1* used in serology were characterized in local parasites circulating before and during the follow-up to confirm that the variants expressed as recombinant antigens match to those which subjects have been exposed to. Ultimately, the relative contribution of antigenic polymorphism, poor immunogenicity, and original antigenic sin (the skew in the specificity of antibodies elicited by exposure to new antigenic variants

due to pre-existing variant-specific responses) were discussed to the observed patterns of antibody recognition of PvMSP-1 variants (Bastos *et al.* 2007).

Filling the remaining gaps between immunology and epidemiology requires a coordinated combination of i) ecologically realistic experiments that measure quantities directly relevant to infection and immune dynamics within hosts and the transmission between them, ii) sophisticated deterministic, stochastic or simulation models that simultaneously account for within- and between-host dynamics, iii) large-scale longitudinal surveys and field studies, iv) statistical methods for analyzing large spatiotemporal and sequence data sets and v) standards and guidelines for future data collection (Hellriegel, 2001). Combining immunology and epidemiology will bring us closer to assessing the actual role of acquired immunity in determining well-characterized epidemiological patterns. This will help to distinguish between infection and disease and to explain their epidemiological patterns. A detailed understanding of naturally acquired and treatment-induced immunity in individuals and populations and of their consequences for parasite transmission is important for planning effective intervention programmes, including resistance management, and for quantifying their success (Hellriegel, 2001).

1.14. 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).

Martinelli *et al.* (2004) demonstrated the use of Linkage Group Selection (LGS) to locate regions in a *Plasmodium* genome that contains genes encoding target antigens of strain-specific immunity. Regions in the genome of the malaria parasite *P. chabaudi chabaudi* were located, which contained genes controlling strain-specific immunity against the disease causing blood stages of the parasites. These regions include a well defined selection valley whose focus lies on the gene encoding the malaria vaccine candidate antigen MSP-1. As of the equivalence of the genetic features of their life cycles, LGS will be equally applicable to other Apicomplexan parasites, including pathogens such as the *Theilerias*, *Babesias* and *Eimerian* parasites of domestic animals (Martinelli *et al.* 2004).

Results from Buckling and Read (2001) on *P. chabaudi* may have implications for understanding the epidemiology of human malaria and the effects of immune intervention. Partial immunity significantly reduced transmission, even though there were compensatory increases in gamytocytogenesis, implying that even partially protected vaccines might be of value in reducing transmission. Strain-specific immunity might also have a large impact on transmission, not as a consequence of strain-specific reductions in the densities of asexual parasites, but because of strain-specific reductions in gametocyte infectivity (Buckling and Read, 2001).

Using a transfection approach to create isogenic parasite lines that differ only by expression of different *Pfama-1* alleles, Healer and co-workers (2004) conclusively demonstrated that differences in antibody-mediated inhibition of merozoite invasion resulted from polymorphisms within this gene, confirming previous studies using different laboratory isolates (Hodder *et al.* 2001; Kennedy *et al.* 2002). This study addresses how genetic diversity within *ama-1* affects the susceptibility of parasites to invasion-inhibitory antibodies (Healer *et al.* 2004). Further, Xainli *et al.* (2003) demonstrated that concurrent infection with a particular

PvDBP parasite strain appeared to boost strain-specific immunity. In conclusion, it is noteworthy that the number, type, and pattern of delivery of malarial inoculations and the type of malarial endemicity, to which an individual is exposed, must profoundly affect his or her immune status and hence disease status.

1.15. Objectives of the present study

The efficacy of a vaccine is hindered by the polymorphism elicited by the parasite to escape the host immune system, thus the characterization of antigenic diversity among locally prevalence parasite strains will be particularly important in a specific geographical setting. Thus the present study attempted to characterize the prevailing genetic diversity of two major asexual stage vaccine antigens, the PvMSP-1_{42} and the domain II of PvAMA-1 in patients infected with *P. vivax* malaria living in Colombo, a non-endemic area for malaria and from Anuradhapura and Kataragama, two endemic areas for malaria in Sri Lanka, where unstable malaria prevails with low transmission. Further, based on the prevailing genetic diversity, it is essential to evaluate whether sequence differences among each antigen is indicative of functional antigenic polymorphism at the level of antibody responses ensuing strain-specific immunity.

Specific objectives of the present study are as follows:

1. To evaluate the prevailing genetic diversity at the C-terminal of *Pvmsp-1*(*Pvmsp-*1_{42}) and at domain II of *Pvama-1* in field isolates of Sri Lanka and to examine the *P. vivax* population diversity within Sri Lanka and among published worldwide isolates.

2. To evaluate the total and isotype specific antibody against seven overlapping synthetic peptides spanning domain II of PvAMA-1, using the homologous serum samples of the panel of clinical isolates analyzed under objective one.

3. To assess the possible strain-specific immunity to PvAMA-1 domain II, PvMSP-1_{42} and PvMSP-1_{19} by aligning amino acid sequences with the homologous total (IgM + IgG) antibody responses assayed against the seven PvAMA-1 domain II peptides and the recombinant PvMSP-1_{42} and PvMSP-1_{19} proteins.

CHAPTER 2

Materials and Methods

2.1. Study sites

Following approval by the ethics review committee of the University of Colombo, Sri Lanka (EC/04/103), pre-treatment blood samples were collected with informed consent from microscopically confirmed *P. vivax* infected patients (age >15 yr) presenting from December 1999 – January 2001, and December 2002 - June 2004 from patients living in three areas of the island, where unstable malaria prevails with low transmission, Colombo, a non-endemic area for malaria, Anuradhapura and Kataragama, two endemic areas for malaria (Figure 2.1).

Of the three climatic zones in Sri Lanka, Anuradhapura and Kataragama are sited in the dry zone, which covers majority of the country. It receives less than 2000 mm of rain annually, primarily from October to January during the northeast monsoon. Colombo, in the wet zone, receives an average rain fall of more than 2500 mm per year from October to January and May to July during the southwest monsoon (Konradsen, *et al.* 2000). In endemic areas malaria transmission is favourable due to the average temperature of around 25°C and high humidity of around 75-80% (Nagendran, *et al.* 1993). However, recent statistics show a slight increase in incidences in the past years, and several incidences in few places of the wet zone of Sri Lanka (Galappaththy, G. N. L., Personal communication), where, with exceptionally low rainfall, breeding opportunities are created in streams and rivers. However, abundant rain fall that occurs through out the year in the wet zone is not conductive to the breeding of the malaria vector and hence malaria incidence remains less in that particular zone (Rajendram & Jayewickreme, 1951).

Patients from both endemic areas are mainly engaged in agriculture and live in small houses in villages that surround the main city (Konradsen *et al.* 2000). Though, Colombo is an urban city where majority are engaged in private and government occupations, most patients from both the endemic and non-endemic areas attend treatment at government health care institutions, which provide health services at no cost. Patients from the three areas were self selected as they presented themselves to a clinic for diagnosis.

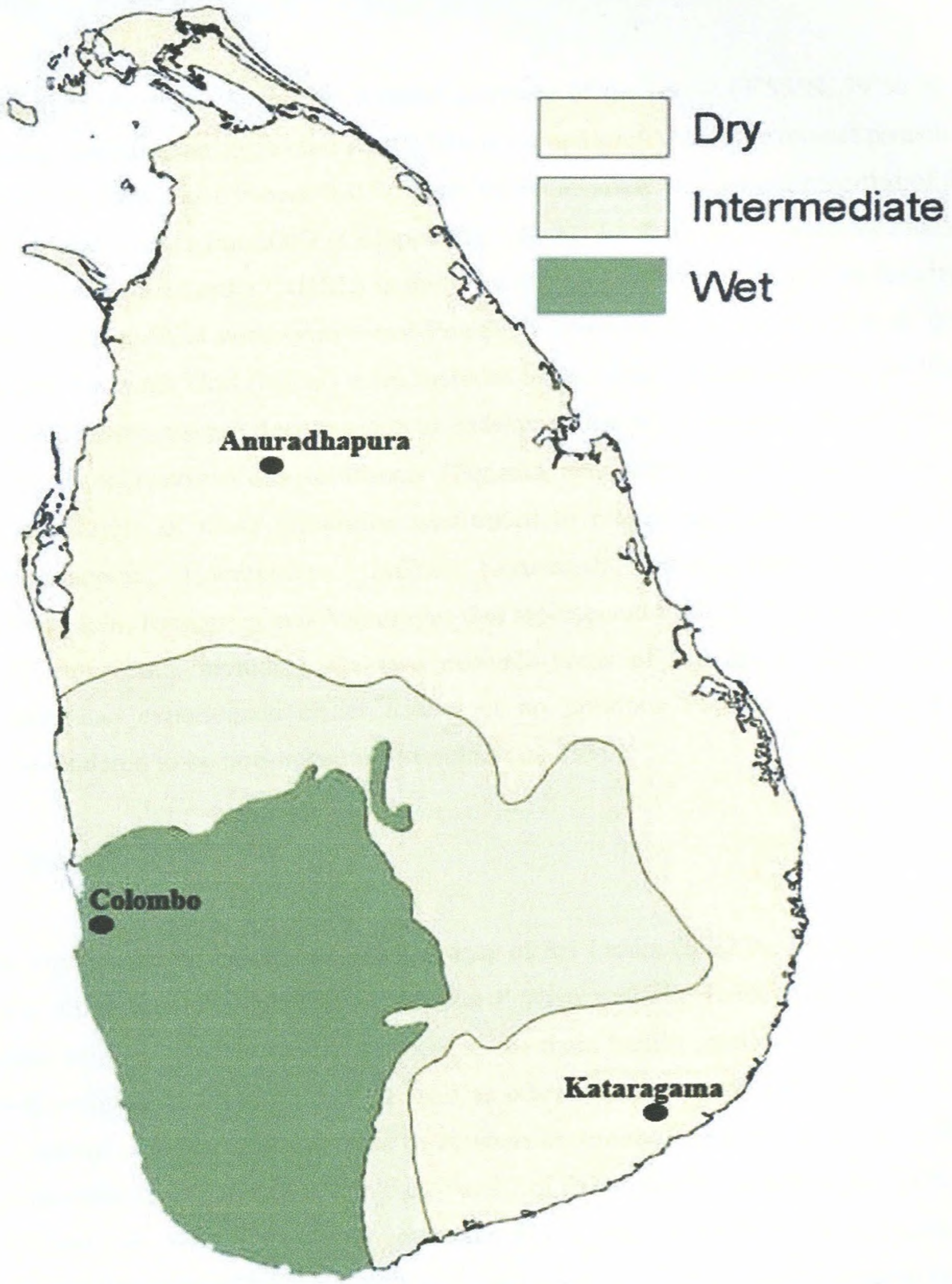


Figure 2.1. Map of Sri Lanka showing the locations of the three study areas and the climatic zones relating to malaria transmission in the island

2.1.1. Colombo

Colombo, regarded as the main city in the Western province of the island (7°55'N, 79°50'E), a malaria free area, where according to the Anti Malaria Campaign (AMC) the annual parasite incidence per 1000 in 2008 was between 0-0.009 and only 5 *P. vivax* cases were recorded of 8 malaria positive blood smears inn 2009 (Galappaththy, G. N. L., Personal communication). The National Hospital of Sri Lanka (NHSL) is the main hospital where patients from the city and around present themselves with symptoms. Positively screened *P. vivax* patients by the staff of the Malaria Research Unit (MRU) were included in this study. These patients residing in Colombo contracted the disease during visits to endemic areas of the country with *P. vivax* transmission due to occupational commitments (Fonseka and Mendis, 1987; Briet *et al.*, 2003), where the origin of these infections was traced to eleven administrative districts (Ampara, Anuradhapura, Hambantota, Jaffna, Kurunagala, Monaragala, Mullaitivu, Pollonnaruwa, Puttalum, Ratnapura and Vavuniya) that represented both the intermediate and the dry zone of the island, including the two endemic areas of this study (Figure 2.2). Furthermore, they had experienced either a few or no previous malaria infections and therefore were considered to be non-immune (Mendis *et al.* 1990).

2.1.2. Anuradhapura

Anuradhapura, situated in the North Central province of Sri Lanka (8°22'N, 80°20'E) is the main city of this province and is an endemic area for *P. vivax* malaria (Briët *et al.* 2003). The General Hospital of Anuradhapura (GHA) serves as the main health institute of this region. Malaria patients residing in both the city as well as other suburbs present to the GHA for treatment. The annual parasite incidence due to *P. vivax* in Anuradhapura from 2004 – 2008 had drastically dropped from 0.6-1 to 0.001-0.005 and 7 of the 8 positive blood smear were *P. vivax* (Galappaththy, G. N. L., Personal communication). This demonstrates the greater variability of the transmission of the vector through out the region. The Malaria Research Unit (MRU) of the Faculty of Medicine, University of Colombo maintains a malaria clinic at the GHA, where patient records of 1995 and onwards are available as information. Patients with laboratory confirmed acute *P. vivax* infections were included in this study.

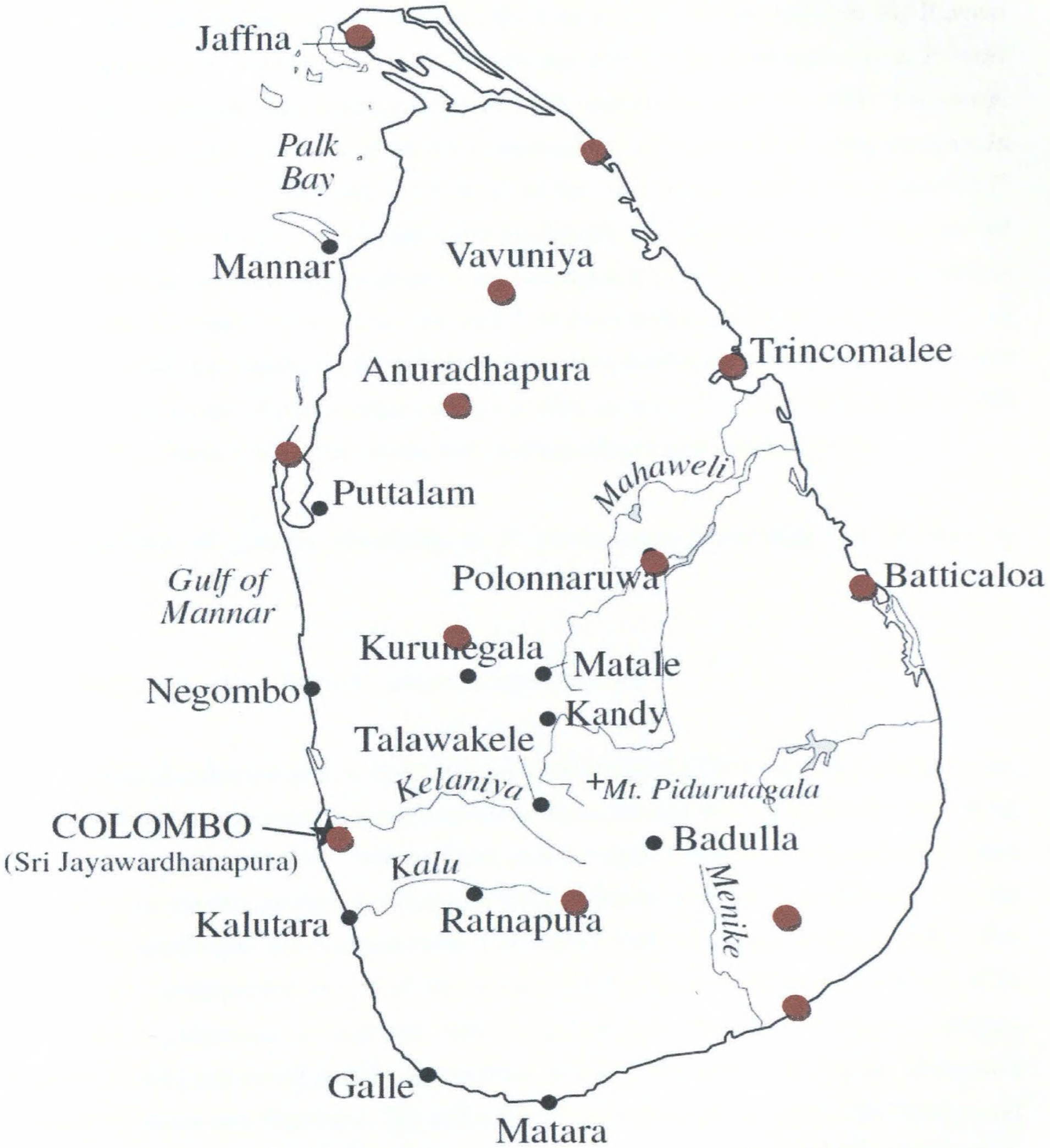


Figure 2.2. Map of Sri Lanka showing the districts (in red) where patients from non-endemic Colombo had contracted the disease

2.1.3. Kataragama

Kataragama (6°25'N, 81°20'E), situated in the low country coastal plains of the Monaragala district in the Uva province of the south of Sri Lanka is seasonally endemic for *P. vivax* malaria. Though, both parasite species have been prevalent in the Kataragama area, *P. vivax* had a higher inoculation rate during the period 1998 (Karunaweera *et al.* 1998). The annual parasite incidence due to *P. vivax* from the Monaragala district was 1.6 in 2004, however, in 2008 it decreased to 0.01-0.05 and in 2009 all of the 97 positive blood smears were of *P. vivax* (Galappaththy, G. N. L., Personal communication). The blood samples were collected from patients who presented themselves to the Kataragama base hospital and to the Malaria Research Station (MRS) operated by the MRU of the Faculty of Medicine, University of Colombo. A study by Mendis *et al.* (1990) contained information on host factors (e.g. number of previous malaria infections, occupation, age, gender, etc.) of 3735 individuals infected with *P. vivax* malaria since 1986 within seven surrounding villages in a computer data.

2.2. Analysis of genetic diversity at *P. vivax ama-1* domain II and *msp-1*$_{42}$ genes

2.2.1. DNA extraction from *P. vivax* infected blood

Five ml of blood collected in to a final concentration of 2mM EDTA via vein 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 was centrifuged at 1000 x g for 10 minutes. The pellet 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 alchohol (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).

2.2.2. Parasite genotyping and identification of single-clone infections

Parasite stages that infect the human host are haploid and thus, each contains only one 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 from the present study, each of the DNA samples were genotyped at the polymorphic *msp-3α* locus using a combined nested polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) strategy described by Bruce *et al.* (1999).

2.2.2.1. Amplification of the *P. vivax msp-3α*

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, oligonuleotide primers were used at a final concentration of 0.5 µM (Integrated DNA Technologies, USA) and each of the four deoxynucletoide triphosphates at 0.2 mM (Promega, USA). The final $MgCl_2$ (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). 1X Tris-Acidic acid-EDTA (TAE) buffer (Appendix III) was used as the running buffer. Sizing of products was carried using a standard curve drawn from DNA markers run adjacently (0.1 µg/lane, 1 kb ladder; Promega, USA).

2.2.2.2. Restriction fragment length polymorphism analysis of *Pvmsp-3α*

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

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 µl of each digested product was run on a 1.5% agarose gel (Sigma Chemicals, USA) containing 0.5 µg/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 µg/lane, 100 bp ladder; Promega, USA).

Any multiple clone isolates 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 (Wickramarachchi *et al.* 2010).

2.2.3. PCR amplification of the domain II of *Pvama-1*

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 µl, using 1 µl 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 µM (Integrated DNA Technologies, USA) and each of the four deoxynucleotide triphosphates at 0.2 mM (Promega, USA). The final $MgCl_2$ 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 dimmers, a hot start PCR was performed, 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.

The amplified product, containing 2 µl of the PCR product mixed with 1µl of the Blue/Orange loading dye (Promega, USA) 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). The amplified PCR products were sized using a standard curve drawn from a 100 bp DNA marker run adjacently (Promega, USA).

2.2.4. PCR amplification of *Pvmsp-1*$_{42}$

Amplification of the 1162 bp length *Pvmsp-1*$_{42}$ 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, oligonuleotide primers were used at a final concentration of 0.2 µM (Integrated DNA Technologies, USA) and each of the four deoxynucletoide triphosphates at 0.1 mM (Promega, USA). The final $MgCl_2$ (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. The resultant PCR products were visualized by UV illumination following electrophoresis on 1% agarose gels (Promega, USA) containing 0.25 µg/ml of ethidium bromide (Promega, USA, 10 mg/ml). The amplified PCR products were sized using a standard curve drawn from a 1 kb DNA marker run adjacently (Promega, USA).

2.2.5. Purification and quantification of the PCR products

Both the *Pvama-1* domain II and *Pvmsp-1*$_{42}$ 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, while 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 that lacked primer dimmers 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 an additional 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 -20°C until further use.

Two micro liters of the purified DNA mixed with 1 µl of the tracking dye was 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.

2.2.6. Sequencing of the purified PCR products

Sequencing of each of the purified PCR products of *Pvama-1* domain II and *Pvmsp-1*$_{42}$ 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 *Pvmsp-1*$_{42}$ gene, and for both the genes a fourfold coverage was attempted for at least 10 isolates.

2.2.7. Editing and assembling of sequence products

Each forward and reverse sequence from the same isolate was assembled using SeqMan programme and initially, was edited manually using visual analysis of the corresponding chromatograms into a full-length contig that contains a single amino acid sequence without any stop codons (DNAStar Inc, Madison, WI, USA). The consensus sequences were opened via EditSeq and translated to its consensus sequence. The reading frame of each sequence was determined using the National Center for Biotechnology Information (NCBI) Open Reading Frame (ORF) finder (www.ncbi.com). The sequences obtained through the ORF finder were copied to the MEGA version 4.1 software (Tamura *et al.* 2007) for sequence alignment. Nucleotide differences were checked for possible insertions or deletions using the appropriate chromatograms. The 41 PvAMA-1 domain II and the 95 PvMSP-1_{42} sequence data were submitted to Gene Bank database under the accession numbers GU433899 – GU433939 and GU175174 – GU175268, respectively.

2.2.8. Genetic diversity parameters and phylogenetic analysis

Several measures of genetic diversity were employed in this study. DnaSP version 4. 90.1 package (Rozas *et al.* 2003) was used to calculate i) π: an estimate of the average number of substitutions between any 2 sequences, assuming the sample is random (all sites, synonymous and replacement substitutions were considered in this calculation); ii) S: the number of polymorphic sites in the sample, which is dependent on the sample size and length of sequence; and iii) H: the number of haplotypes, as transitions are generally more frequent than transversions

Evidence of positive selection was determined by comparing the difference between the rate of non-synonymous and synonymous mutations (Dn-Ds). Significance of the difference can be calculated by the Z test. The rates of substitutions were estimated using the Nei and Gojobori's method (Nei and Gojobori, 1986) as implemented in the MEGA 4.1 program (Tamura *et al.* 2007). Standard error was determined by 1000 bootstrap replications. A significant excess of non-synonymous mutations compared to synonymous mutations indicate positive natural selection whereas excess synonymous mutations indicate negative or purifying selection (Nei and Gojobori, 1986; Escalante *et al.* 2004).

Tests of evolutionary neutrality were also performed using Tajima's D statistic (Tajima, 1989). This test is based on the neutral model prediction that there are two different ways of estimating the expected heterozygosity under the neutral model from a random sample of alleles obtained from a population (summarized in the parameter θ). One of the estimates is based on the number of segregating sites and the other on pairwise differences among alleles. If these two estimates of θ show a statistically significant discrepancy, then the null hypothesis, the neutral model, is rejected (Escalante *et al.* 2004).

The assumption of neutrality was also tested by the McDonald and Kreitman test (McDonald and Kreitman, 1991), using *P. cynomolgi* gene (Dutta *et al.* 1995) as the out group as it is closely related to *P. vivax* (88% sequence identity). The MK test compares patterns of polymorphism (diversity within species) and divergence (substitutions between species) by subdividing them into synonymous and non-synonymous substitutions. The synonymous and non-synonymous substitutions of divergence are compared with those of polymorphism by using a 2 x 2 contingency table. Under neutrality the ratio of synonymous substitutions between species should be the same as the ratio of synonymous substitutions within species. However, when the ratio of non-synonymous to synonymous mutations is significantly greater between species than within *P. vivax* sequences, it is an indicator of positive selection occurring. However, when the ratio of non-synonymous to synonymous mutations is significantly smaller between species than within *P. vivax*, it is suggestive of purifying selection operating to reduce the number of non-synonymous substitutions between species.

Evidence of recombination in terms of recombination parameter (R) and minimum number of recombination events (Rm) were also measured using DnaSP. Linkage disequilibrium (LD) between different polymorphic sites was computed in terms of R^2 index.

The intra and inter-population genetic diversity was calculated using the DnaSP version 4.90.1 where the difference between populations was measured by the fixation index (F_{ST}). F_{ST} is the ratio of the sum of the estimated variances of the haplotype frequencies due to differences in haplotypes in different populations and those due to differences among the different populations to the total variance in haplotype frequency. 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 that an allele is found in only one population (Cole-Tobian and King, 2003).

Phylogenetic analysis was used to investigate the relatedness of *Pvmsp-1*$_{42}$ and *Pvama-1* domain II alleles in different parts of the world, separately. Two phylogenetic trees were drawn; i) including only the amino acid haplotypes obtained in each gene and ii) including all available global isolate sequences in the gene bank of *Pvama-1* domain II and *Pvmsp-1*$_{42}$ using the Neighbor-Joining method with the (Saitou and Nei, 1987) with the Kimura 2 parameter model as implemented in the MEGA 4.1 program (Tamura, *et al.* 2007). The phylogeny confidence was assessed by using 1000 bootstrap pseudo-samples. We used *P. cynomolgi ama-1* domain II and *msp-1*$_{42}$ gene sequences as out groups and the cut off was 70% in the condensed tree.

Since *ama-1* sequences of both *P. vivax* and *P. falciparum* exhibit significant homology between them, the polymorphisms were compared in these orthologous molecules among the Sri Lankan isolates. Here, domain II polymorphisms were compared between these two species referring to the polymorphic sites depicted by Chesne-Seck, *et al.* (2005).

2.2.9. Prediction of T cell epitopes at PvAMA-1 domain II and PvMSP-1$_{42}$

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 PvAMA-1 domain II and PvMSP-1$_{42}$ 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). SYFPEITHI T cell prediction software (Rammensee *et al.* 1999) established the theoretical binding of predicted T cell epitopes of 15-mer residues to the four HLA alleles, taking into account only epitopes with a score above 25 (Saravia *et al.* 2008). Allocation of scores is based on the frequency of the respective amino acid in natural ligands, T-cell epitopes or binding peptides (Rammensee *et al.* 1999). The ProPred MHC class II binding peptide prediction server predicted T cell epitopes of 9-mer residues, where the 1st residue was an obligatory P anchor residue, which is considered for high affinity binding. Amino acids M(Met), F(Phe), I(Ile), L(Leu), V(Val), W(Trp) and Y(Tyr) are found at P1 in significant proportion of high affinity binders therefore according to Sturniolo *et al.* (1999), only these amino acids are allowed at P1 position.

2.2.10. Prediction of B cell epitopes on PvAMA-1 domain II and PvMSP-1_{42}

Using the BcPred software (EL-Manzalawy *et al.* 2008), linear B cell epitopes spanning the PvMSP-1_{33} of the 27 PvMSP-1_{42} amino acid haplotypes and domain II of PvAMA-1 was predicted. This linear B cell prediction tool consists of the epitope-based predictors with specified epitope lengths. Therefore, 12 amino acid length epitopes with a specificity of 75% were predicted. The score of each peptide represents the prediction of the mean score of several combined propensity scales (e.g., flexibility or hydrophilicity).

2.2.11. Homology modelling of the amino acid haplotypes of PvAMA-1 domain II and PvMSP-1_{42}

Percentage homology of the 21 amino acid haplotypes of PvAMA-1 domain II was obtained with respect to the recombinant protein which represented the Salvador I strain that was used to analyse the antibody responses of these patients (Wickramarachchi *et al.* 2006), which was also the predominant haplotypes in the clinical isolates of Sri Lanka. In the case of PvMSP-1_{42}, the percentage homology of the 27 amino acid haplotypes were obtained with respect to the Belem strain, as the recombinant protein used represented the Belem strain (Wickramarachchi *et al.* 2007). A sequence identity matrix was performed using the BioEdit software (Hall, 1999).

2.3. Serum

The collected blood samples from the MRU of the three areas were subjected to serum separation by Dr. Thilan Wickramarachchi and were stored at -20°C. These serum samples were kindly provided by Prof. Preethi V. Udagama-Randeniya of the Department of Zoology, University of Colombo. As normal healthy controls, residents in Colombo who were never exposed to malaria and were not experiencing any illness at the time were chosen for blood collection and serum was separated by Dr. Upadari Dissanayake. Serum samples that were positive for total (IgM + IgG) anti-AMA-1 antibodies assayed against the recombinant PvAMA-1 protein (PV66/AMA-1) as recorded previously (Wickramarachchi, 2005) were selected as test serum for the current study.

2.4. Host Factors

The age and the clinical histories including the number of past malaria infections suffered by each patient were recorded from verbal statements obtained by a questionnaire (Karunaweera *et al.* 1998).

2.5. Synthetic Peptides

Seven synthetic peptides were designed to the domain II of PvAMA-1 Salvador I strain with overlapping 4-6 amino acids (Figure 2.3). The seven peptides were demarcated by predicted linear B cell epitopes obtained by BCPREDS Server 1.0 (EL-Manzalawy *et al.* 2008). The designed peptides were synthesized by Mimotopes Pty Ltd, Australia and had undergone Electrospray Mass Spectrometry (ESMS) and Reverse Phase High Performance Liquid Chromatography (RP-HPLC) to test for quality.

Each peptide was first mixed with 50 µl of nuclease free (NF) water. If the peptide was dissolved, the final volume was brought up to 1ml with NF water. If the peptide was not dissolved in NF water, the amino acid sequence of the peptide was calculated for the overall net charge. If the sequence had a positive or a negative net charge, dilute acetic acid or dilute aqueous ammonia was added and sonicated to improve solubility. In this study, peptides P01, P02, P04 and P06 were dissolved in NF water, while P03 and P07 were dissolved in 0.5% liquid ammonia, and P05 was dissolved in 0.1% acetic acid, and the final volume in all peptides were brought up to 1 ml with NF water.

2.6. Analysis of the antibody responses to the overlapping synthetic peptides

2.6.1. Indirect microplate ELISA to demonstrate total (IgM + IgG) antibody responses

The total (IgM + IgG) antibody responses against the seven synthetic overlapping peptides were assayed by an indirect microplate enzyme linked immunosorbant assay (ELISA) as previously described (Wickramarachchi, 2005) with modifications. Each test serum sample was assayed against the peptide in duplicate as well as with normal human serum as control.

Checkerboard titrations were carried out to determine the optimal dilutions of reagents to be used in this ELISA to increase specificity and the sensitivity of the assay. The optimum antigen concentrations for each of the seven peptides were determined by titrating each antigen separately for total (IgM + IgG) antibody responses (Appendix IV).

To determine the optimum serum concentrations for each peptide, 20 samples of immune serum which were positive with high antibody responses to the total (IgG + IgM) antibody responses against PvAMA-1 ectodomain (Wickramarachchi *et al.* 2006) were pooled, while serum collected from 30 normal healthy donors were pooled and used as the negative control. For each peptide, curves were derived from the absorbance values of the pooled immune serum dilutions by titrating sera from 1:10 to 1:200. The optimum dilution of serum was found to be 1:50 for peptides P01-P04, P06 and 1:25 for P05 and P07.

Horseradish peroxidase (HRP) linked rabbit anti-human IgM and IgG antibodies (DAKO Immunoglobulins, Denmark) was used as the secondary antibody (Conjugate) and the optimum concentration was titrated accordingly (Appendix VI).

Sodium chloride (VWR International Ltd, UK) concentrations ranging from 0.5M to 1.5M was tested in washing buffer, and 1M was selected as the working concentration. Tween 20 (Sigma Chemicals, USA) concentrations ranging from 0.1% - 1.5% were tested in washing buffer and a final concentration of 0.2% was selected. Further, 5% non-fat milk (Anchor, New Zealand) was selected as the optimum concentration to prepare the blocking buffer. To maximize the specific binding of high affinity as well as low affinity antibodies the optimal incubation after each step and the final colour development time and temperature were determined.

Hundred micro litres of each peptide (in appropriate concentration) mixed in 1X phosphate buffer (PB), pH 7.4 (Appendix VII) 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 200 µl f blocking buffer (Appendix VIII). Plates were incubated at room temperature for two hours in a humid chamber and flick washed four times in washing buffer (Appendix IX) 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 X) 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 IgM and IgG 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 containing OPD (O-phenylenediamine) (Sigma Chemicals, USA) at a concentration of 1 mg/ml in substrate buffer (Appendix XI) with 0.03% hydrogen peroxide (H_2O_2) (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 XII), after which the optical density (OD) was measured at 490 nm in an automated ELISA reader (Model 680, BioRad International, USA).

As the OD values of the 30 normal controls by ELISA were normally distributed and they were age matched with test sera samples, the cut off value 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 the cut off value was considered to be positive responses.

2.6.2. Antibody sandwich ELISA to demonstrate the isotype (IgM) and (IgG) antibody responses

An antibody sandwich ELISA (Wickramarachchi, 2005) was performed for two peptides (P04 and P05) using subclass specific mouse anti-human monoclonal antibodies (Mab) (Sigma Chemicals, USA) for the quantification of IgM and total IgG isotypes.

Checkerboard titrations were carried out to determine the optimal dilutions of reagents to be used in this assay. The optimum antigen concentrations for the two peptides were determined by titrating each antigen against each antibody subclass (Appendix IV).

Optimal serum dilutions were selected by titrating against each Mab. The optimal dilution for the two sub classes were selected from each standard curve drawn for the two peptides. A serum dilution of 1:15 was selected for both peptides.

Optimal dilutions of each anti-human mouse monoclonal (Appendix V) and HRP linked goat anti-mouse IgG antibodies (Sigma Chemicals, USA) (Appendix VI) were determined by checkerboard titration.

Sodium chloride (VWR International, UK) in the washing buffer was selected to be 1M. Tween 20 (Sigma, Chemicals, USA) concentration was adjusted to 1% and 5% non-fat milk (Anchor, New Zealand) was used to prepare the blocking buffer.

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 each serum sample (that was positive for total (IgM + IgG) antibodies against P04 and P05), 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 containing OPD (Sigma Chemicals, USA) at a concentration of 1 mg/ml in substrate buffer with 0.03% H_2O_2 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).

2.7. 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) (Center for disease control and prevention, USA and World Health Organization, Switzerland) computer software packages.

Proportion of responders (total and isotype specific) using independent variables were tested using the Chi square 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 with 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 trends as appropriate. The significant level was set as $P<0.05$.

2.8. Assessing strain-specific immune responses against domain II of PvAMA-1, PvMSP-1_{42} and PvMSP-1_{19}

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.

Similarly, to evaluate possible strain-specific immune responses against PvMSP-1_{42}, seventy two of the 95 *P. vivax* MSP-1_{42} 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-1_{42} antibody responses previously assayed by an indirect ELISA against recombinant protein PvMSP-1_{42} (Wickramarachchi *et al.* 2007). The recombinant protein PvMSP-1_{42} 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-1_{19}.

CHAPTER 3

Genetic Diversity of domain II of *Plasmodium vivax* Apical Membrane Antigen-1 in *P. vivax* infected patients from Sri Lanka

3.1. Introduction

Studies on population diversity of malaria parasites have practical significance on the development of strategies for disease control, including vaccine development (Cui *et al.* 2003). Moreover, the characterization of genes responsible for resistance to therapeutic agents by both *Plasmodium falciparum* and *P. vivax* depends on a thorough knowledge of each parasite's genetic diversity in the natural population (Hughes and Verra, 2001). The primary factors affecting genetic diversity at such loci are natural selection (Escalante *et al.* 2004), and genetic drift (Holsinger & Weir, 2009) where the latter reflects population history (Greenberg *et al.* 2008). Thus, knowledge of the parasite's population history and its genetic diversity is important for an in depth understanding of the epidemiology of malaria, and the potential response of the parasite to therapeutic strategies.

An effective malaria vaccine should elicit protective immune responses that either destroy the parasite or its infected host cell, or inhibit a function that is crucial to its survival (Hoffman *et al.* 2002). Pathology of malaria is a consequence of the parasitemia which develops through cyclical asexual replication of *Plasmodium* parasites in the patient's red blood cells (RBC). The malaria parasite's ability to first recognize and then to invade RBC's is vital to the disease process, and parasite molecules involved in these recognition and invasion steps are widely agreed to be targets for prophylactic immunization (Mitchell *et al.* 2004). 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).

3.1.1. Apical Membrane Antigen-1

One such antigen that showed promise as a vaccine candidate is the Apical Membrane Antigen-1 (AMA-1). First identified as an invariant *Plasmodium knowlesi* merozoite surface antigen (Reviewed in Remarque *et al.* 2007), AMA-1 is believed to be unique to apicomplexa

with orthologues existing in two apicomplexan parasites, *Toxoplasma gondii* (Hehl *et al.* 2000) and *Babesia bovis* (Gaffar *et al.* 2004), and it derives from a single essential gene present in all *Plasmodium* species (Waters *et al.* 1990). 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).

3.1.2. Structure and sequence of AMA-1

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). Apical Membrane Antigen-1 has been cloned and sequenced from a number of *Plasmodium* species including *P. vivax* (Kocken *et al.* 1999), *P. falciparum* (Peterson *et al.* 1989), *P. knowlesi* (Waters *et al.* 1990), *P. fragile* (Peterson *et al.* 1990), and *P. chabaudi* (Marshall *et al.* 1989). The *ama*-1 gene comprises an N-terminal ectoplasmic region, a single transmembrane region and a small cytoplasmic domain. Sixteen invariant Cysteine (Csy) 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 Pizzaro 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 (*P*lasminogen, *A*pple, *N*ematode), 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.* 2008b) suggesting that functional constraints may operate to prevent antigenic variation in this surface exposed segment.

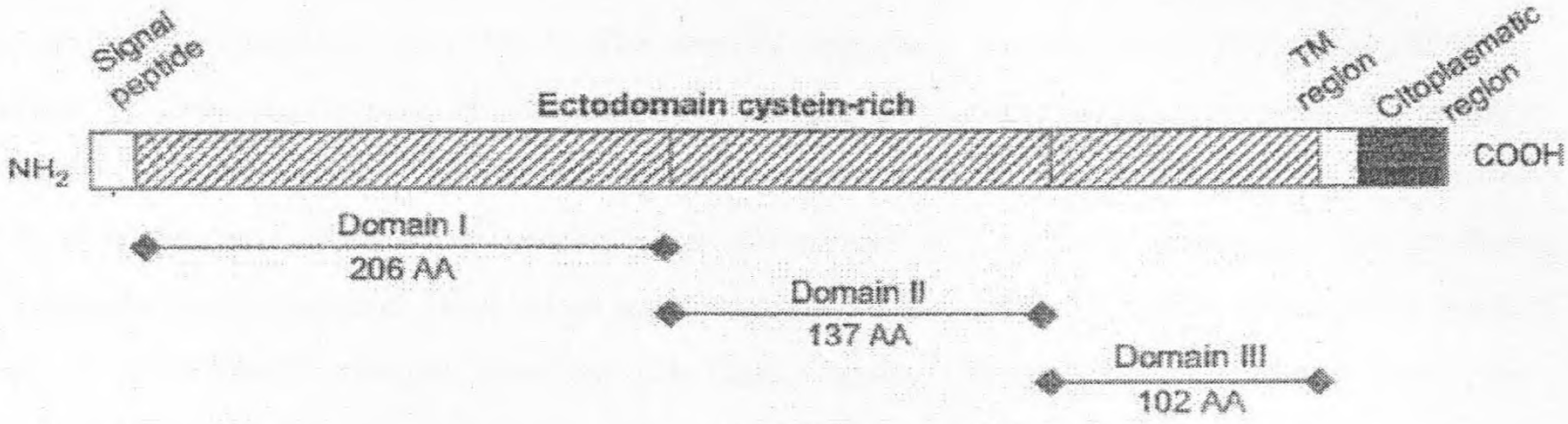


Figure 3.1. Schematic diagram of the structure of *Pv*AMA-1. Each of the three domains separately (DI, DII and DIII)
(Adopted from Múrfalo *et al.* 2008)

Several *Plasmodium* AMA-1 ectoplasmic sequences, which are highly conserved, were compared by Chesne-Sek *et al.* (2005), with the secondary structure components observed in the crystal structure of PvAMA-1. The overall sequence identity was 39%, with 40% in domain I, 38% in domain II and 33% in domain III for the sequences chosen for this comparison. A number of polar and apolar interactions in the PvAMA-1 crystal structure were made by residues that were invariant or conserved in character between *Plasmodium* orthologues and included both inter and intradomain contacts. A large number of polar contacts were made between invariant side chains and the polypeptide backbone. These are believed to contribute equally to the structural similarity of AMA-1 among different species (Chesne-Seck *et al.* 2005). AMA-1 lacks the sequence repeats and size polymorphisms found in other malaria vaccine candidates (Cortés *et al.* 2003).

3.1.3. Function of AMA-1

Failure to disrupt AMA-1 in *Plasmodium* (Triglia, *et al.* 2000) and *T. gondii* (Hehl *et al.* 2000) strongly suggested that AMA-1 has vital functions. Direct evidence implicating AMA-1 in host cell invasion comes from studies with transgenic *T. gondii* and a conditional AMA-1-knockout phenotype (Mital *et al.* 2005). In the absence of AMA-1, *T. gondii* tachyzoites cannot invade host cells. Rhoptry secretion is impaired, whereas micronemes function normally, suggesting a two-stage attachment model in the second stage of which TgAMA-1 is involved in regulating rhoptry secretion. Further studies with these parasites led to the identification of three rhoptry proteins associated with TgAMA-1, one of which interacted at the moving junction during the invasion process (Alexander *et al.* 2005). Other Apicomplexa were homologous for these proteins, suggesting that apicomplexan-invasion machinery is conserved. Long thought to be involved in red blood cell invasion by *Plasmodium* merozoites, AMA-1 might function during reorientation of the merozoite when the apical organelles are aligned with the erythrocyte membrane (Mitchell *et al.* 2004) and could also be involved in liver cell invasion (Silvie *et al.* 2004). In the rodent malaria *Plasmodium yoeli*, DI/II was implicated in erythrocyte binding through COS-7 cell-surface expression assays (Fraser *et al.* 2001). Furthermore, structural features pointing to a possible receptor-binding role included a) the presence of PAN-folding motifs and the high proportion of unstructured regions, suggestive of potential involvement in binding to protein or carbohydrates (Fink, 2005); and b) the presence of a hydrophobic cleft comprising several residues indicative of possible receptor-binding sites (Bai *et al.* 2005).

Although the functional implications of AMA-1 shedding are unknown, it might point to a role for AMA-1 to interact directly with red blood cell surface components by the formation of a molecular complex that must be shed before the parasite can enter the red blood cell. Some antibodies that prevent invasion also interfere with shedding, raising the possibility that shedding is essential for invasion and presenting one possible mechanism by which anti-AMA-1 antibodies exert invasion-inhibitory activity (Dutta *et al.* 2005b).

3.1.4. Natural Processing of AMA-1

The AMA-1 molecule of *P. falciparum* (PfAMA-1) is synthesized as an 83 kDa precursor (Howell *et al.* 2001). In common with most micronemal proteins, AMA-1 is processed proteolytically, such that, around the time of merozoite release and red blood cell invasion, the prosequence is cleaved away. In *P. falciparum* AMA-1 (PfAMA-1), the 83 kDa precursor protein (PfAMA-1_{83}) is converted to PfAMA-1_{66} (Narum & Thomas, 1994), an event that proceeds to circum-merozoite relocalization of AMA-1. At or around the point of invasion, the bulk of the ectodomain is then shed quantitatively in the form of a PfAMA-1_{48} component by the membrane-bound subtilisin-like 'sheddase' PfSUB2 (Howell *et al.* 2003; Harris *et al.* 2005b). Proteolytic cleavage of the AMA-1 ectoplasmic region also occurs in *P. knowlesi* as reported by Deans *et al.* (1984) and is likely to take place in other *Plasmodium* species, although this has not been experimentally confirmed. Cleavage of the AMA-1 ectoplasmic region from the tachyzoites was also observed for TgAMA-1 (Hehl *et al.* 2000), although this occurs at an intramembrane site (Howell *et al.* 2005). Antibodies that prevent cleavage of AMA-1 in *P. falciparum* inhibit invasion, suggesting that shedding may be a prerequisite for invasion (Dutta *et al.* 2005b). Chesne-Seck and colleagues (2005) observed two partial cleavage sites by N-terminal sequencing of the soluble recombinant PvAMA-1 ectoplasmic region expressed in *Pichia pastoris*, both presumably mediated by a protease(s) from *P. pastoris*. One site occurred at the domain III region and the second partial proteolysis site in recombinant PvAMA-1 occurred at the domain II loop (Pizzaro *et al.* 2005). However, due to the extensive hydrogen bonds, protection against proteolysis was anticipated at this peptide bond. Thus, if AMA-1 in *P. vivax* (and other *Plasmodium* species) is cleaved like PfAMA-1 from the parasite surface during erythrocyte invasion, it was suggested that the conformation of this region of the protein differs from that observed in the crystal structure at least during the proteolysis step. This hypothesis was proven incorrect by Igonet and colleagues (2007) by demonstrating SDS/polyacrylamide gels performed under reducing conditions. The recombinant protein recovered from crystals of the PvAMA-1 ectoplasmic region was

partially proteolysed, proving thus, that the cleavage occurring in the disordered loops in the crystal structure of the complete PvAMA-1 ectoplasmic region does not affect the overall integrity of the native conformation.

3.1.5. Polymorphism of AMA-1

Antigenic diversity is one of the main mechanisms used by malaria parasites to evade the host immune system, and it is believed to be one of the major hurdles confronting the development of an effective malaria vaccine (Cortés *et al.* 2003). In mice, immunization with a recombinant protein containing, the two epidermal growth factor (EGF) like domains of MSP-1 or with the AMA-1 ectodomain, conferred protection against homologous but not heterologous sporozoite challenge (Crewther *et al.* 1996; Renia *et al.* 1997). Hence detailed genotyping to determine which of the polymorphisms allow evasion of vaccine-induced protective immune responses, together with knowledge of the sequences found in the population, may aid the development of a future AMA-1 based vaccine that would induce an immune response effective against most AMA-1 variants (Cortés *et al.* 2003).

In all cases where the sequence from different isolates of the same species was examined, there are regions of the gene where many apparently unlinked single base substitutions occur (Gunasekera *et al.* 2007). This results in many potential alleles, making these genes useful markers for typing parasite populations (Figtree *et al.* 2000).

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 & Saul, 1994; Figtree *et al.* 2000; Han *et al.* 2002; Chung *et al.* 2003; Grynberg *et al.* 2007 & 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).

Grynberg *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 Grynberg *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 (Grynberg *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.

In a study in Papua New Guinea, certain polymorphism frequencies differed between symptomatic and asymptomatic cases, suggesting PfAMA-1 to be a determinant of morbidity (Cortés *et al.* 2003). In particular, strong age dependence was observed in asymptomatic individuals in the distribution of residues at most of the positions at which imbalances between symptomatic and asymptomatic infections occurred. This finding suggested that there is an age dependent selection against parasites with certain residues at particular AMA-1 positions, consistent with age dependent strain-specific immunity against these positions being effective in parasite growth.

3.1.6. Polymorphism in PfAMA-1 and PvAMA-1

The sequence of PfAMA-1 from the FVO strain was modelled on the PvAMA-1 (Sal I) crystal structure (58.9% sequence identity) by Chesne-Seck *et al.* (2005) and sequence differences between these two species were distributed over the entire ectoplasmic region (Polley and Conway, 2001; Garg *et al.* 2007; Gunasekera *et al.* 2007; Thakur *et al.* 2008b). As shown for *P. vivax ama-1* gene, the predominance of non-synonymous over synonymous mutations in the *Pfama-1* gene implies that sequence diversity is selected for, and maintained, to counter the host's immune response (Polley and Conway, 2001). Although polymorphic sites were distributed throughout the length of the ectoplasmic region, domain I was most variable in *P. falciparum* (Polley and Conway, 2001, Garg *et al.* 2007). The regions under selective pressure for *P. vivax* polymorphism may differ from those of *Pfama-1*. Studies showed domain I to be polymorphic (Figtree *et al.* 2000; Grynburg *et al.* 2008). However, the first comprehensive study amplifying the entire ectoplasmic region of *Pvama-1* was carried out by Gunasekera and group (2007) where four overlapping templates were sequenced to generate a single consensus sequence. This analysis revealed that the signature of diversifying selection in the Sri Lankan isolates was seen most strongly in domain II of *Pvama-1* where the non-synonymous substitutions were high relative to synonymous substitutions. This was further supported by a study carried out by Thakur *et al.* (2008b) using Indian isolates. Most of the polymorphic sites for *P. falciparum* were dimorphic, with some being trimorphic and tetramorphic. A single heptamorphic site was exclusive for *P. falciparum* however, the corresponding site of *P. vivax* was conserved (Chesne-Seck *et al.* 2005). Using the crystal structure of PvAMA-1, the three-dimensional distribution of polymorphic sites in *P. falciparum* AMA-1 was modelled (Chesne Seck *et al.* 2005). All polymorphic sites were surface exposed and the distribution of the PfAMA-1 polymorphic sites was highly biased to one side of the molecule. A similar observation was seen after modelling the polymorphic sites of PvAMA-1 (Gunasekera *et al.* 2007; Thakur *et al.* 2008b), suggesting that this moiety of the ectoplasmic region may be more exposed to the exterior on the parasite surface and/or less susceptible to functional constraints.

3.1.7. Objective of the study

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, this study was undertaken to analyze the prevailing genetic diversity of *P. viviax ama-1* domain II in individuals infected in Sri Lanka and to predict possible T and B cell epitopes that might influence in a protective antibody response against PvAMA-1 domain II.

3.2. Research design

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 the Kataragama isolates and labelled as isolates originating from the endemic area (EA). Based on the sequences obtained, the objectives of the analysis were:

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 Sri Lanka, and to compare the obtained data with previously published domain II sequences from geographically diverse *P. vivax* populations.

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 the *Pvama-1* domain II gene 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.

3.3. Results

PCR amplification of parasite DNA from patients resulted in a single band of 792 bp corresponding to 306 – 1098 bp of the *Pvama-1* Salvador I sequence. The sequence of these amplicons yielded a 411 bp region, corresponding to 619 –1029 bp of the Sal I strain, encoding up to 136 amino acids of the domain II region, that was selected after excluding primer regions and adjacent bases which did not give uniformly reliable sequence reads. The results were analyzed and compared, using the previously reported *P. vivax* Salvador I (*Pvama-1* Sal I) sequence as the reference sequence (Accession no. Y16950, Kocken *et al.* 1999).

3.3.1. 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 (π = 0.0105 S.D. = 0.0007) compared to EA (π = 0.0088 S.D. = 0.0006) (Table 3.1) 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 3.1). 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 3.1).

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 3.2).

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.

3.3.2. Genetic diversity of *Pvama-1* 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 (Hd) of 0.928 ± 0.015 S.D. (Table 3.1). Comparison of these results with previously published isolates from India (Thakur *et al.* 2008b) indicated that the *ama-1* domain II gene in Sri Lankan isolates were highly diverse. Analysis with the sliding window option revealed nucleotide diversity ranging from 0.007 - 0.026. Maximum diversity was observed between nucleotide positions 869 – 1029, which represented the latter part of domain II (Figure 3.2).

Table 3.1. 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 3.2. McDonald-Kreitman analyses of *Pvama-1* Domain II between *P. vivax* and *P. cynomolgi* across different countries

	Polymorphic changes within *P. vivax*		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

The difference between Dn and Ds in the entire Sri Lankan population illustrated a significant positive selection acting on this domain (Dn-Ds= 0.0075± 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 3.1). Data obtained by the McDonald Kreitman test which is summarized in Table 3.2, 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 (Rm) was 4, whereas the recombination parameter (C) between adjacent sites and per gene was 0.1483 and 60.8 respectively. The Rm value in the 64 Sri Lankan isolates was similar to the 3 recombination events that occurred within 61 Indian isolates, while the Rm 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 Venezuela (Ord *et al.* 2008; Thakur *et al.* 2008b). However, the recombination parameter between adjacent sites was 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 (Figure 3.3). Low recombination parameter value (C = 0.1483) for the *Pvama-1* domain II, further suggested meagre meiotic recombination occurring in this gene in the local population.

A moderate (F_{ST} = 0.1-0.15) genetic differentiation was evident between Sri Lanka and Venezuela (F_{ST} value = 0.1472), whereas similar F_{ST} values were calculated between Sri Lanka, India (F_{ST} value = 0.0692) and India, Venezuela (F_{ST} value = 0.0641).

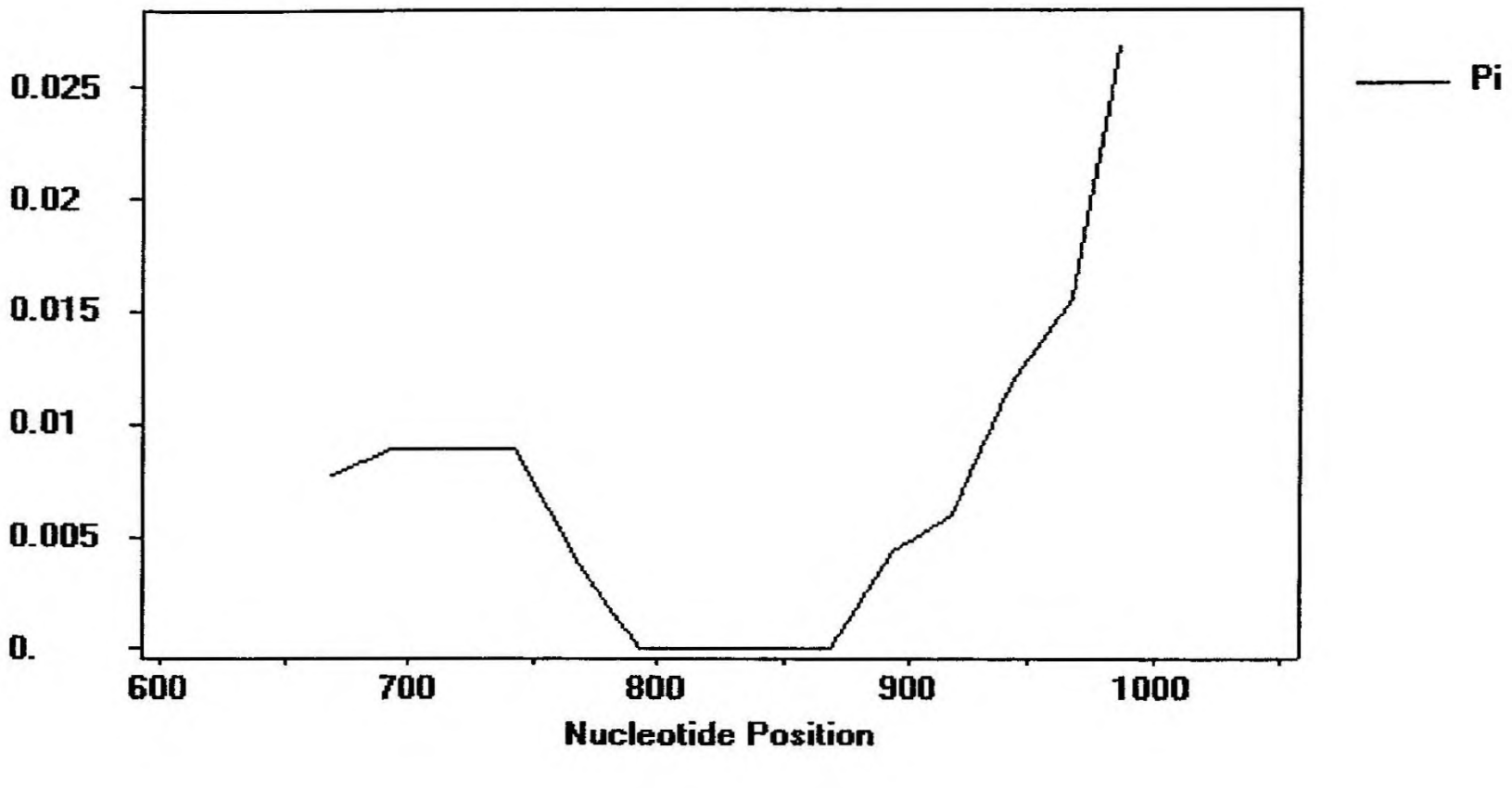


Figure 3.2. Sliding window plot of nucleotide diversity per site (π) comparing the level of genetic diversity at *Pvama-1* domain II

The π values were calculated on DnaSP with window length 100 bp and step size 25 bp. The nucleotide positions correspond to the *Pvama-1* Salvador I strain (Accession No. Y16950).

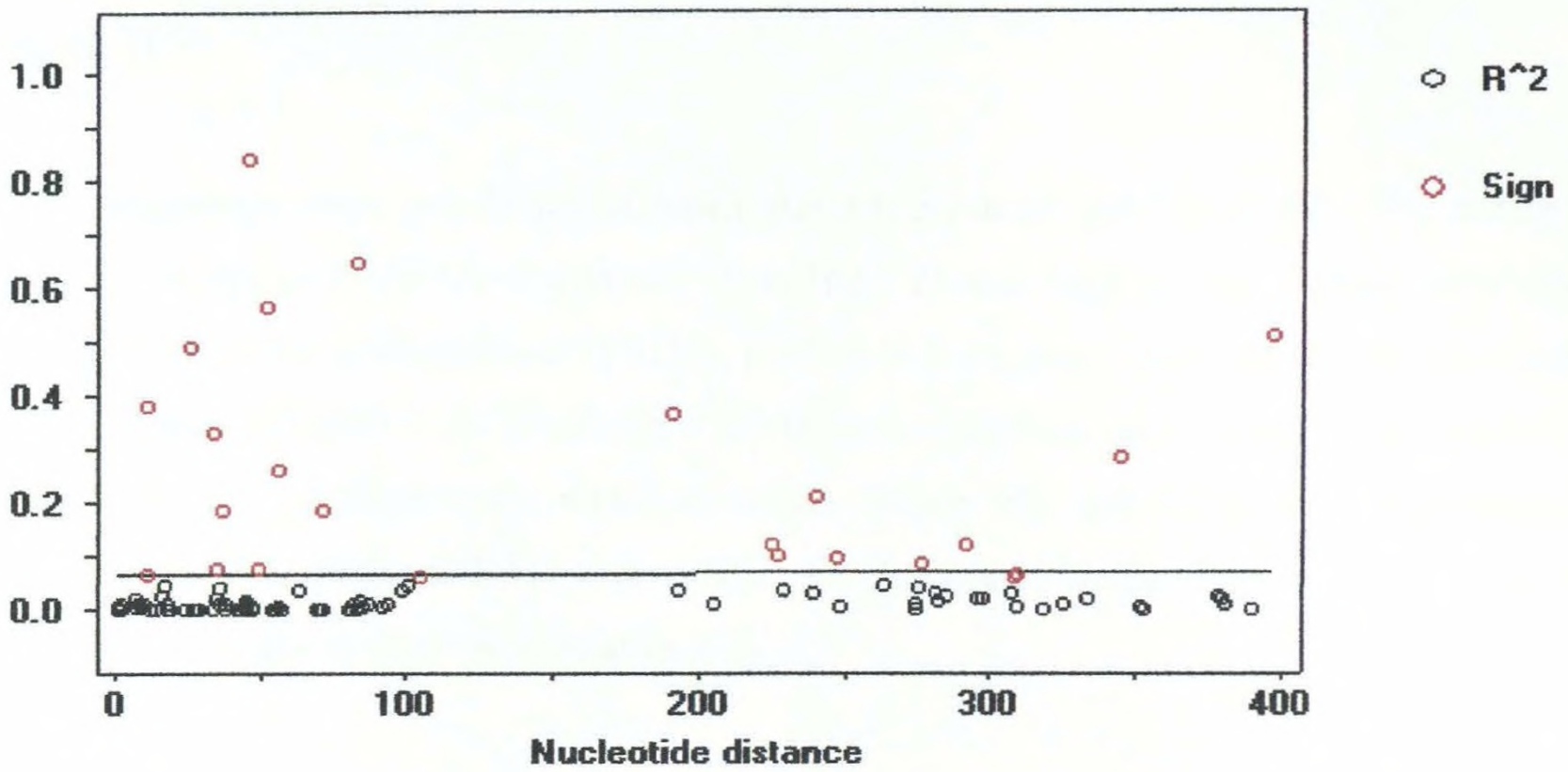


Figure 3.3. Linkage Disequilibrium (LD) plot showing non-random association between nucleotide variants in the 64 Sri Lankan isolates at different polymorphic sites of *Pvama-1* domain II

Sites with significant linkage ($P<0.05$) are shown as red open circles

3.3.3. 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-1 domain II 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 3.3). 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 3.3).

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 3.4). 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 3.4).

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 3.3). 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 3.3). Haplotype H8 which was represented by a single Sri Lankan isolate from Kataragama was similar to sixteen Venezuelan isolates (Ord *et al.* 2008) (Table 3.3 & 3.4). An isolate from the Philippines

(PH84) published by Cheng & Saul (1994), was similar to an Indian isolate, while the Brisbane isolate (PVQ) (Cheng & Saul, 1994) was included among 8 previously published Indian isolates (Thakur *et al.* 2008b). Only a single haplotype was distinctive to Venezuela, while 8 haplotypes were exclusive to India as reported by Thakur *et al.* (2008b). Of the 21 amino acid haplotypes observed from the 64 Sri Lankan isolates, 10 were unique to the island (Table 3.3). Further, as 4 of these haplotypes (H5, H12, H13, and H20) were previously published by Gunasekera *et al.* (2007), 6 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 3.3).

Comparison of these 22 polymorphic nucleotide positions with other countries resulted in 12 amino acid changes (Table 3.3). Of the 12 polymorphic amino acid positions identified in this and previous studies (Ord *et al.* 2008; Thakur *et al.* 2008b), 4 were trimorphic (K352E/N, Q380R/K, L384P/R and E385Q/D), while the rest of the codons displayed only two alternative amino acid residues and thus were dimorphic (Table 3.3). A single amino acid change found in PV4 and PV12 Indian isolates and 21 Thai isolates, where Valine (V) was replaced by Glutamate (E) at position 382 was not recorded in the Sri Lankan and Venezuelan isolates. Moreover, position 382 was the only amino acid position where all the Sri Lankan isolates were similar to isolates from the Philippines (PH84), Brisbane (PVQ) and Sal-I. At positions 370 and 371, where non-synonymous changes occurred from Phenylalanine (F) to Leucine (L), and Isoleucine (I) to Phenylalanine (F) in 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 370 and 371 were exclusively distinct to Sri Lanka while position 382 was unique to India.

Haplotype	Sri Lanka N=64	Philippine	Brisbane	India N=60	Venezuela N=73	253	257	278	352	368	370	371	380	382	383	384	385
P. vivax 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 253 - 285 correspond to domain II of PvAMA-1 Salvador I sequence (Accession No Y16950) H1-H21 represented Sri Lankan, H22-H32 Indian and H33 Venezuelan haplotypes

[a] - haplotypes exclusive to Sri Lanka

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

[c] – haplotypes exclusive to Venezuela (Ord *et al.* 2008)

Table 3.4. 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)

3.3.4. Phylogenetic analysis of *Pvama-1* in geographically different countries

The phylogenetic tree constructed using the amino acid haplotypes of PvAMA-1 domain II showed significant divergence of each haplotype (Figure 3.4). Haplotype H1 which was identical to the Sal I strain was mapped closer to the Sal I strain, while H2 and H11 which differed from H1 by only a single amino acid substitution at amino acid positions 368 and 384, respectively, were closely related to H1. Haplotypes H4, H12 and H10, H13 were the only two clusters observed with a cut off of 95 and 98%, respectively. Due to a single amino acid substitution occurring at amino acid positions 370 from Phenylalanine to Leucine (F/L) and 383 from Aspartic acid to Valine (D/V), H12 differed from H4 and H10 differed from H13 in the two clusters, respectively (Figure 3.4).

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 3.5). 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 4.3). The PVQ Brisbane isolate was clearly clustered between several Venezuelan isolates (Figure 3.5).

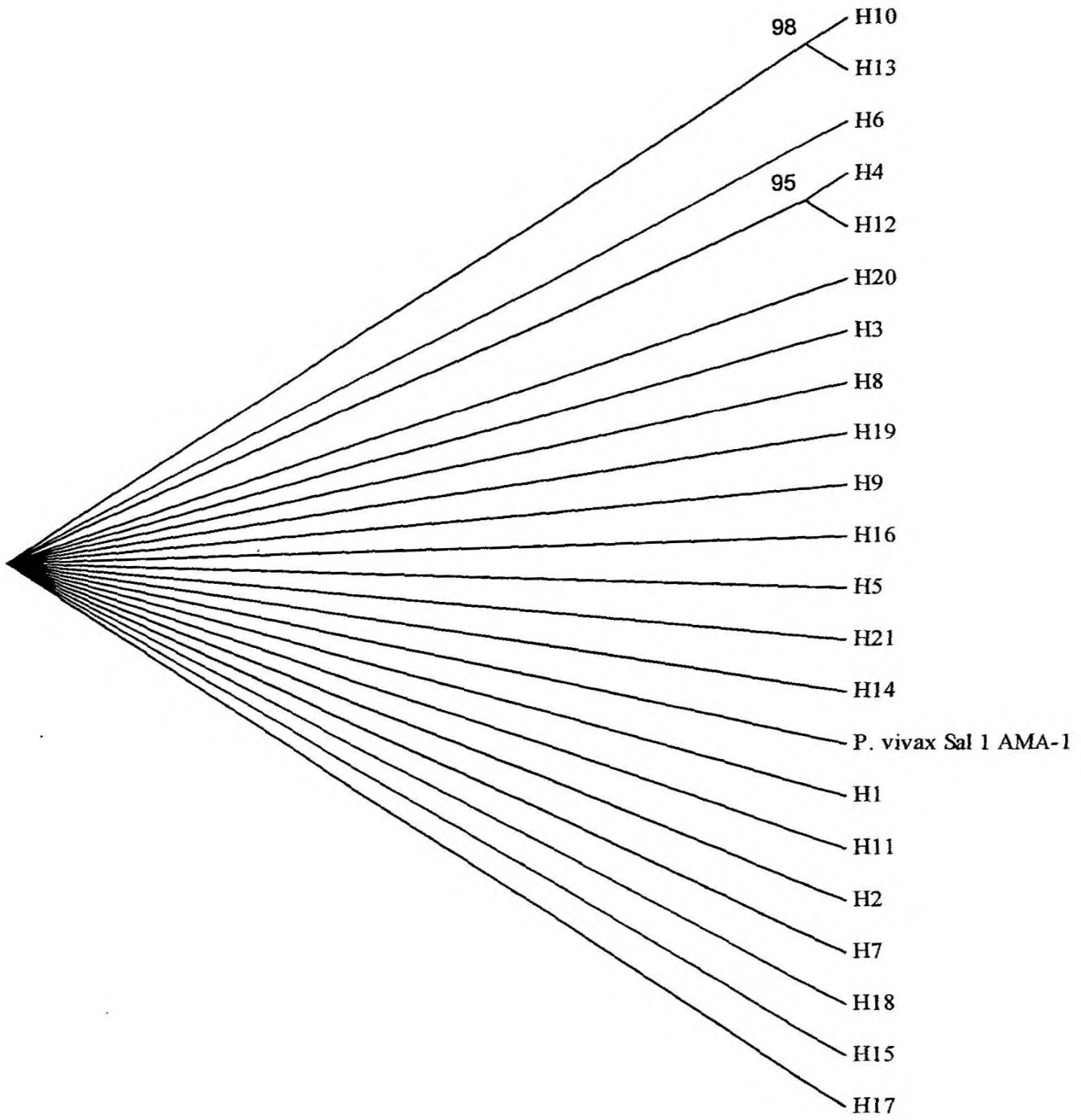


Figure 3.4. Phylogenetic tree consisting of the 22 amino acid haplotypes of the Sri Lankan population using the Neighbor-Joining method, with Kimura 2 parameter

Bootstrap values above 70% are displayed on the tree.

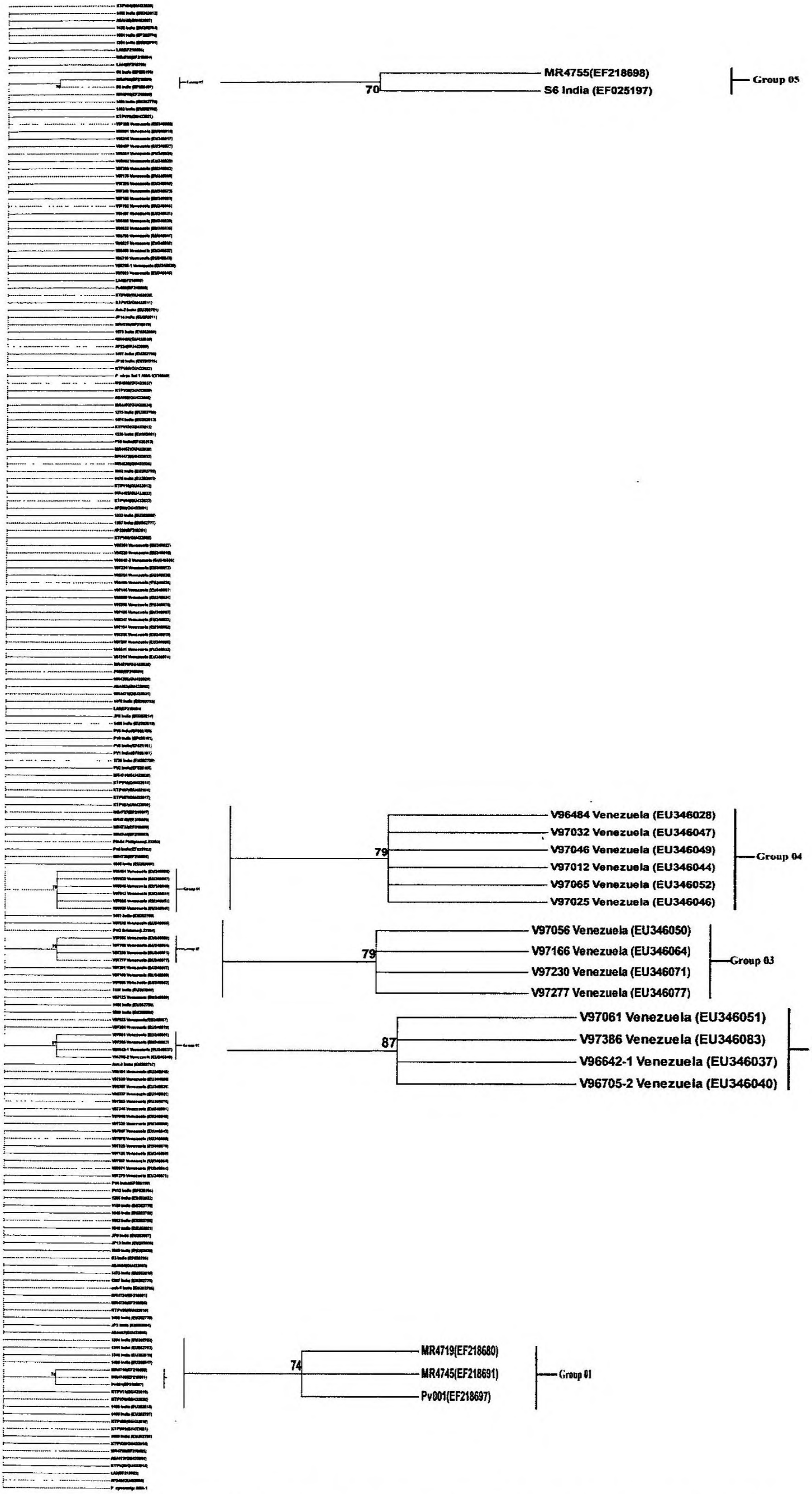


Figure 3.5. Phylogenetic tree of *Pvama-1* domain II using Kimura 2 parameter

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 100 pseudo-replications, only boot strap values above 70% are displayed on the tree

3.3.5. Comparison of polymorphism in PfAMA-1 and PvAMA-1

AMA-1 is polymorphic both in *P. falciparum* (Kocken *et al.* 2000) and *P. vivax* (Figtree *et al.* 2000). An analysis was carried out to compare polymorphism of domain II between both these orthologues by obtaining the sequence of *P. falciparum* AMA-1 (Accession no AJ277646) and comparing it with sequences from Sri Lanka isolates together with isolates from other geographic locations.

Predominance of non-synonymous over synonymous mutations was detected in both Pf and PvAMA-1 domain II. Comparison of the domain II sequence of PfAMA-1 and PvAMA-1 revealed a sequence identity of 63%. Of the 11 polymorphic sites observed for PfAMA-1, five were common to PvAMA-1 with polymorphism occurring at codons 253, 277, 352, 380 and 384 (Table 3.5), while rest of the sites which were polymorphic in *P. falciparum* were conserved in *P. vivax* isolates from Sri Lanka, India and Venezuela (Table 3.5). Polymorphic site at amino acid position 308 which was trimorphic in *P. falciparum* was dimorphic in *P. vivax* isolates, whereas at position 407 the reverse was observed (Table 3.5).

3.3.6. Linear B and T cell epitope prediction on domain II of PvAMA-1

Six linear B cell epitopes were predicted in the domain II of PvAMA-1 with the use of the BcPred software (EL-Manzalawy *et al.* 2008) (Figure 3.6). This linear B cell prediction tool consists of the epitope-based predictors with specified epitope lengths. Therefore, 12 amino acid length epitopes with a specificity of 75% were predicted. The score of each peptide represents the prediction of the mean score of several combined propensity scales (e.g., flexibility or hydrophilicity).

Subsequently, 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, which are among the 10 most prevalent in Afro-American and Asian populations (Southwood *et al.*, 1998). Five T cell epitopes, constituting different sequence lengths were distributed on these four HLA alleles (Figure 3.6).

Table 3.5. Comparison of polymorphic sites in domain II of PfAMA-1 and PvAMA-1

PfAMA-1		PvAMA-1				
				Sri		
Codons[a]	Mutations	Codons[b]	Mutations	Lanka[c]	India[d]	Venezuela[e]
308	**E/Q/K**	**253**	**G/E**	**54/10**	**71/1**	**73/-**
325	H/D/R	270	Y	-	-	-
330	S/P	275	E	-	-	-
332	**N/I**	**277**	**E/K**	**32/32**	**11/61**	**73/-**
393	H/R/Y	338	K	-	-	-
395	K/R	340	R	-	-	-
404	R/T	349	S	-	-	-
405	K/E	350	V	-	-	-
407	**Q/H**	**352**	**K/E/N**	**51/7/6**	**64/5/3**	**61/-/12**
435	**I/N/T**	**380**	**Q/K/R**	**53/7/4**	**69/2/1**	**64/9/-**
439	**H/N/D**	**384**	**L/P/R**	**31/16/17**	**17/28/27**	**31/42/-**

Polymorphic sites were detected with respect to domain II of PfAMA-1

The polymorphic amino acid residues common (5 such positions) in both PfAMA-1 and PvAMA-1 are boldfaced.

[a]- Codon number correspond to PfAMA-1 Sal I strain (Accession no AJ277646)

[b]- Codon number correspond to PvAMA-1 Sal I strain (Accession no Y16950; Kocken *et al.* 1999)

The number of sequences in each country contributing to each polymorphic substitution at each site in PvAMA-1 is given separately in the same order as the amino acid substitutions themselves.

[c]- The current study

[d]- Data from Thakur *et al.*, 2008b

[e]- Data from Ord *et al.*, 2008

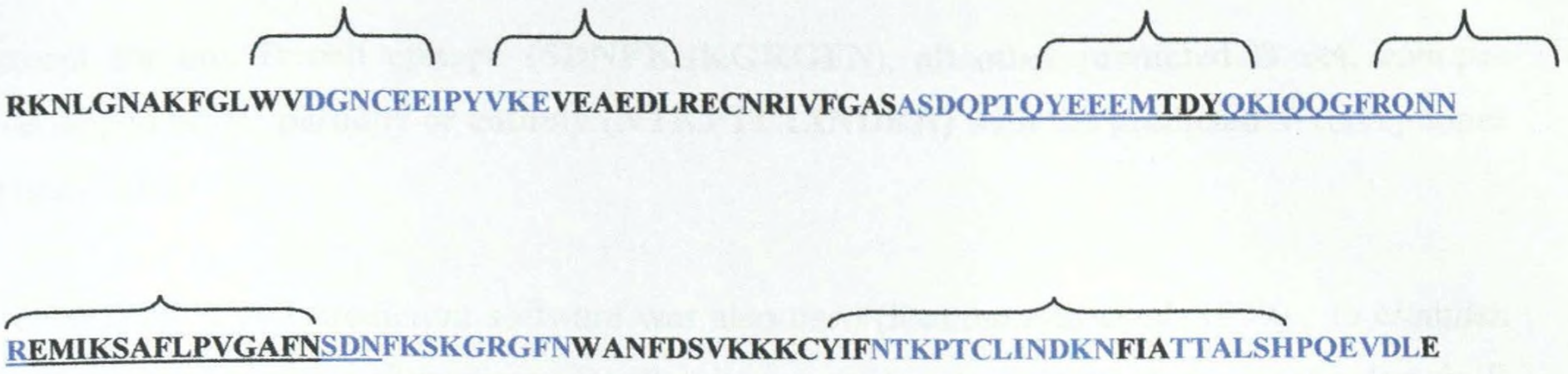


Figure 3.6. Predicted B and T cell epitopes spanning domain II of PvAMA-1

Predicted epitopes highlighted in blue correspond to B cell epitopes. Epitopes in brackets correspond to T cell epitopes, which were manifested to four HLADRB alleles representing; HLA-DRB1*0101, DRB1*0401, DRB1*0701 and DRB1*1101 (Southwood *et al.* 1998) by the ProPred MHC class II binding peptide prediction serer (Singh and Raghava, 2001). Underlined sequence represents the domain II loop (Chesne-Seck *et al.* 2005).

Except for one B cell epitope **(SDNFKSKGRGFN)**, all other predicted B cell epitopes overlapped either partially or entirely **(NTKPTCLINDKN)** with the predicted T cell epitopes (Figure 3.6)

SYFPEITHI T cell prediction software was also used (Rammensee *et al.* 1999) to establish the theoretical binding of predicted T cell epitopes of 15-mer residues spanning the domain II to the four HLA alleles, taking (Table 3.6) into account only epitopes with a score above 25 (Saravia *et al.* 2008). Allocation of scores is based on the frequency of the respective amino acid in natural ligands, T-cell epitopes, or binding peptides (Rammensee *et al.* 1999).

According to the SYFHEITHI prediction, 4 and 3 T cell epitopes were predicted to HLA-DRB1*0101 and DRB1*0401 alleles respectively, with scores between 25- 33. HLA-DRB1*0701 allele consisted of only a single epitope with a score of 26, while epitopes with a score above 25 were non-existing to the HLA-DRB1*1101 allele. HLA DRB*0701 and HLA DRB*0101 alleles both consisted of the T cell epitope CYIFNTKPTCLINDK (Table 3.6). Further six epitopes predicted by ProPred MHC class II peptide binding server to the four HLA alleles were also recorded from the SYFPEITHI server, whereas as only a single epitope GFNWANFDDSVKKKCY corresponding to amino acid position 341 were unique to latter server (Table 3.6).

Certain polymorphic substitutions at domain II which fall upon the predicted T and B cell epitopes might have an effect on the binding capacity of these epitopes to HLA alleles (Figure 3.7 and Table 3.7). Figure 3.7 A, 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 288 with a substitution from Glutamic acid (E) / Glycine (G) was only observed in the epitope predicted to HLA-DRB1*0401, while the remaining two substitutions at positions 370 from Leucine (L) / Phenylalanine (F) and 371 from Phenylalanine (F) / Isoleucine (I) were observed in 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 3.7B). The substitution from L/F at amino acid position 370 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 3.6B7B). Due to substitutions from L/F and F/I at positions 370 and 371 a new T cell epitope was predicted to HLA-DRB1*1101, while the predicted T cell epitope to HLA-DRB1*0101 was lost (Figure 3.7B).

Table 3.6. SYFPEITHI predicted epitopes of the PvAMA-1 domain II region binding to the HLA-DRB1*0101, HLA-DRB1*0401 and HLA-DRB1*0701 molecules

A) SYFPEITHI predicted PvAMA-1 domain II epitopes binding to HLA-DRB1*0101

Amino Acid Position	Predicted Epitope	Score
321	K S A F L P V G A F N S D N F	33
268	I P Y V K E V E A E D L R E C	32
280	R E C N R I V F G A S A S D Q	27
354	C Y I F N T K P T C L I N D K	25

B) SYFPEITHI predicted PvAMA-1 domain II epitopes binding to HLA-DRB1*0401

295	P T Q Y E E E M T D Y Q K I Q	28
341	G F N W A N F D S V K K K C Y	28
361	P T C L I N D K N F I A T T A	26

C) SYFPEITHI predicted PvAMA-1 domain II epitope binding to HLA-DRB1*0701

354	C Y I F N T K P T C L I N D K	26

Of the six predicted linear B cell epitopes on domain II of PvAMA-1 for Sal I strain, polymorphic substitutions at amino acid positions 380, Glutamine/Lysine/Arginine (Q/K/R); 383, Aspartic acid/Valine (D/V); 384, Leucine/Proline/Arginine (L/P/R); and 368, Lysine/Isoleucine (K/I) were recorded in two epitopes TTALSHPQEVDL and NTKPTCLINDKN (Table 3.7A). Substitution occurring from Q/K and L/P at amino acid positions 380 and 384 respectively, resulted in the loss of the predicted B cell epitope at amino acid position 373 (Table 3.7B). A single polymorphic substitution from L/R and L/P at position 384 separately decreased the score to 0.598 and 0.646 respectively (Table 3.7.C and D); the polymorphic substitution from Q/K lacked the predicted B cell epitope (Table 3.7E), while the substitution from Q/R decreased the score of the B cell epitope from 0.678 to 0.423 (Table 3.7F). A distinctively polymorphic amino acid position at 383 from D/V increased the score to 1 (Table 3.7G). Nevertheless, while the B cell epitope predicted was lost due to the polymorphism occurring at positions 380 and 383 from Q/K and D/V respectively (Table 3.7H), the score of the predicted B cell epitope increased to 0.979 due to the polymorphism of Q/R and D/V at positions 380 and 383; moreover, previously predicted epitope amino acid position 373, shifted to amino acid position 374 (Table 3.7I). The score also increased in the predicted B cell epitope at position 373 due to the subsequent polymorphisms of D/V, L/P and D/V, L/R at positions 383 and 384 (Table 3.7.J and K), while the predicted epitope at amino acid position 373, shifted to 374 (Table 3.7K).

3.3.7. Homology modelling of the PvAMA-1 domain II

Homology modelling of the PvAMA-1 domain II of the 21 amino acid haplotypes was carried out with respect to the most predominant amino acid haplotype (H1) in the Sri Lankan population. Sequence identity matrix of these amino acid haplotype sequences revealed that all the amino acid haplotypes closely resembled the Sal I strain of PvAMA-1 domain II, as the H1 haplotypes was 100% homologous to the domain II of PvAMA-1 Sal I strain (Figure 3.8). Of the 21 amino acid haplotypes, H6, H7, H9, H12, H13 and H14 representing majority of the haplotypes (29%) were 97% homologous to the domain II of Sal I. Haplotypes H2, H11, H18 had a distinctive homology percentage of 99.2%, while H3, H5, H17 and H19 recorded a 98.5% homology percentage. Though, a 97.8% homology percentage was observed for haplotypes H4, H8, H16, H20, H21, a 96.3% homology percentage was observed for H10 and H15 (Figure 3.8).

A)

DRB1_0101:

RKNLGNAKFGLWVDGNCEEIPYVKEVEAKDLRECNRIVFEASASDQPTQYEEEMTDYQKIQQGFRQNNREMIKSAFLPVGAFNSDNFKSKGRGFNWANFDSVKEKCYIFNTKPTCLINDINLFATTALSHPKEVVPD

DRB1_0401:

RKNLGNAKFGLWVDGNCEEIPYVKEVEAKDLRECNRIVFEASASDQPTQYEEEMTDYQKIQQGFRQNNREMIKSAFLPVGAFNSDNFKSKGRGFNWANFDSVKEKCYIFNTKPTCLINDINLFATTALSHPKEVVPD

DRB1_0701:

RKNLGNAKFGLWVDGNCEEIPYVKEVEAKDLRECNRIVFEASASDQPTQYEEEMTDYQKIQQGFRQNNREMIKSAFLPVGAFNSDNFKSKGRGFNWANFDSVKEKCYIFNTKPTCLINDINLFATTALSHPKEVVPD

DRB1_1101:

RKNLGNAKFGLWVDGNCEEIPYVKEVEAKDLRECNRIVFEASASDQPTQYEEEMTDYQKIQQGFRQNNREMIKSAFLPVGAFNSDNFKSKGRGFNWANFDSVKEKCYIFNTKPTCLINDINLFATTALSHPKEVVPD

B)

DRB1_0101:

RKNLGNAKFGLWVDGNCEEIPYVKEVEAEDLRECNRIVFGASASDQPTQYEEEMTDYQKIQQGFRQNNREMIKSAFLPVGAFNSDNFKSKGRGFNWANFDSVKKKCYIFNTKPTCLINDKNFIATTALSHPQEVDLE

DRB1_0401:

RKNLGNAKFGLWVDGNCEEIPYVKEVEAEDLRECNRIVFGASASDQPTQYEEEMTDYQKIQQGFRQNNREMIKSAFLPVGAFNSDNFKSKGRGFNWANFDSVKKKCYIFNTKPTCLINDKNFIATTALSHPQEVDLE

DRB1_0701:

RKNLGNAKFGLWVDGNCEEIPYVKEVEAEDLRECNRIVFGASASDQPTQYEEEMTDYQKIQQGFRQNNREMIKSAFLPVGAFNSDNFKSKGRGFNWANFDSVKKKCYIFNTKPTCLINDKNFIATTALSHPQEVDLE

DRB1_1101:

RKNLGNAKFGLWVDGNCEEIPYVKEVEAEDLRECNRIVFGASASDQPTQYEEEMTDYQKIQQGFRQNNREMIKSAFLPVGAFNSDNFKSKGRGFNWANFDSVKKKCYIFNTKPTCLINDKNFIATTALSHPQEVDLE

Figure 3.7. 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 originated within the polymorphic sites in domain II of PvAMA-1

Table 3.7. BcPred predicted B cell epitopes with respect to polymorphism

A) BCPred Predictions with the Sal I strain

Position	Epitope	Score
262	DGNCEEIPYVKE	0.931
291	ASDQPTQYEEEM	0.755
373	TTALSHPQEVDL	0.678
332	SDNFKSKGRGFN	0.615
358	NTKPTCLINDKN	0.601
306	QKIQQGFRQNNR	0.468

B) polymorphism of Q/K and L/P at 338, 342

Position	Epitope	Score
262	DGNCEEIPYVKE	0.931
291	ASDQPTQYEEEM	0.755
332	SDNFKSKGRGFN	0.615
358	NTKPTCLINDIN	0.552
306	QKIQQGFRQNNR	0.468

C) polymorphism of L/R at 342

Position	Epitope	Score
262	DGNCEEIPYVKE	0.931
291	ASDQPTQYEEEM	0.755
332	SDNFKSKGRGFN	0.615
373	TTALSHPQEVDR	0.598
358	NTKPTCLINDIN	0.552
306	QKIQQGFRQNNR	0.468

D) polymorphism of L/P at 342

Position	Epitope	Score
262	DGNCEEIPYVKE	0.931
291	ASDQPTQYEEEM	0.755
373	TTALSHPQEVDP	0.646
332	SDNFKSKGRGFN	0.615
358	NTKPTCLINDIN	0.552
306	QKIQQGFRQNNR	0.468

E) polymorphism of Q/K at 338

Position	Epitope	Score
262	DGNCEEIPYVKE	0.931
291	ASDQPTQYEEEM	0.755
332	SDNFKSKGRGFN	0.615
358	NTKPTCLINDIN	0.552
306	QKIQQGFRQNNR	0.468

F) polymorphism of Q/R at 338

Position	Epitope	Score
262	DGNCEEIPYVKE	0.931
291	ASDQPTQYEEEM	0.755
332	SDNFKSKGRGFN	0.615
358	NTKPTCLINDIN	0.552
306	QKIQQGFRQNNR	0.468
373	TTALSHPREVDL	0.423

G) polymorphism of D/V at 341

Position	Epitope	Score
374	TALSHPQEVVLE	1
262	DGNCEEIPYVKE	0.931
291	ASDQPTQYEEEM	0.755
332	SDNFKSKGRGFN	0.615
358	NTKPTCLINDIN	0.601
306	QKIQQGFRQNNR	0.468

H) polymorphism of Q/K and D/V at 338 and 341

Position	Epitope	Score
262	DGNCEEIPYVKE	0.931
291	ASDQPTQYEEEM	0.755
332	SDNFKSKGRGFN	0.615
358	NTKPTCLINDIN	0.601
306	QKIQQGFRQNNR	0.468

I) polymorphism of Q/R and D/V at 338 and 341

Position	Epitope	Score
374	TALSHPREVVLE	0.979
262	DGNCEEIPYVKE	0.931
291	ASDQPTQYEEEM	0.755
332	SDNFKSKGRGFN	0.615
358	NTKPTCLINDIN	0.601

Seq->	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20	H21
H1	ID	0.992	0.985	0.978	0.985	0.970	0.970	0.978	0.970	0.963	0.992	0.970	0.970	0.970	0.963	0.978	0.985	0.992	0.985	0.978	0.978
H2	0.992	ID	0.978	0.970	0.978	0.963	0.978	0.970	0.978	0.970	0.985	0.963	0.978	0.978	0.970	0.985	0.978	0.985	0.978	0.970	0.970
H3	0.985	0.978	ID	0.992	0.970	0.985	0.970	0.992	0.956	0.963	0.978	0.985	0.970	0.970	0.948	0.978	0.970	0.978	0.985	0.978	0.978
H4	0.978	0.970	0.992	ID	0.963	0.985	0.963	0.985	0.948	0.956	0.970	0.992	0.963	0.970	0.941	0.970	0.963	0.970	0.978	0.978	0.970
H5	0.985	0.978	0.970	0.963	ID	0.963	0.970	0.978	0.963	0.956	0.992	0.956	0.963	0.970	0.956	0.978	0.970	0.978	0.985	0.970	0.992
H6	0.970	0.963	0.985	0.985	0.963	ID	0.956	0.978	0.941	0.948	0.963	0.978	0.956	0.970	0.934	0.963	0.956	0.963	0.970	0.992	0.970
H7	0.970	0.978	0.970	0.963	0.970	0.956	ID	0.978	0.963	0.985	0.978	0.956	0.978	0.985	0.970	0.992	0.970	0.978	0.985	0.963	0.978
H8	0.978	0.970	0.992	0.985	0.978	0.978	0.978	ID	0.956	0.963	0.985	0.978	0.970	0.978	0.948	0.985	0.963	0.970	0.992	0.970	0.985
H9	0.970	0.978	0.956	0.948	0.963	0.941	0.963	0.956	ID	0.978	0.970	0.941	0.985	0.963	0.992	0.970	0.956	0.963	0.963	0.948	0.956
H10	0.963	0.970	0.963	0.956	0.956	0.948	0.985	0.963	0.978	ID	0.963	0.948	0.992	0.970	0.985	0.978	0.963	0.970	0.970	0.956	0.963
H11	0.992	0.985	0.978	0.970	0.992	0.963	0.978	0.985	0.970	0.963	ID	0.963	0.970	0.978	0.963	0.985	0.978	0.985	0.992	0.970	0.985
H12	0.970	0.963	0.985	0.992	0.956	0.978	0.956	0.978	0.941	0.948	0.963	ID	0.956	0.963	0.934	0.963	0.956	0.963	0.970	0.970	0.963
H13	0.970	0.978	0.970	0.963	0.963	0.956	0.978	0.970	0.985	0.992	0.970	0.956	ID	0.978	0.978	0.985	0.956	0.963	0.978	0.963	0.970
H14	0.970	0.978	0.970	0.970	0.970	0.970	0.985	0.978	0.963	0.970	0.978	0.963	0.978	ID	0.956	0.992	0.956	0.963	0.985	0.978	0.978
H15	0.963	0.970	0.948	0.941	0.956	0.934	0.970	0.948	0.992	0.985	0.963	0.934	0.978	0.956	ID	0.963	0.963	0.970	0.956	0.941	0.948
H16	0.978	0.985	0.978	0.970	0.978	0.963	0.992	0.985	0.970	0.978	0.985	0.963	0.985	0.992	0.963	ID	0.963	0.970	0.992	0.970	0.985
H17	0.985	0.978	0.970	0.963	0.970	0.956	0.970	0.963	0.956	0.963	0.978	0.956	0.956	0.956	0.963	0.963	ID	0.992	0.970	0.963	0.963
H18	0.992	0.985	0.978	0.970	0.978	0.963	0.978	0.970	0.963	0.970	0.985	0.963	0.963	0.963	0.970	0.970	0.992	ID	0.978	0.970	0.970
H19	0.985	0.978	0.985	0.978	0.985	0.970	0.985	0.992	0.963	0.970	0.992	0.970	0.978	0.985	0.956	0.992	0.970	0.978	ID	0.978	0.992
H20	0.978	0.970	0.978	0.978	0.970	0.992	0.963	0.970	0.948	0.956	0.970	0.970	0.963	0.978	0.941	0.970	0.963	0.970	0.978	ID	0.978
H21	0.978	0.970	0.978	0.970	0.992	0.970	0.978	0.985	0.956	0.963	0.985	0.963	0.970	0.978	0.948	0.985	0.963	0.970	0.992	0.978	ID

Figure 3.8. Identity matrix generated for the 21 amino acid haplotypes of PvAMA-1 domain II among Sri Lankan *P. vivax* infected isolates

The amino acid sequences were compared with the predominant amino haplotypes H1 of *P. vivax* domain II.

3.4. Discussion

The diversity of the *ama-1* domain II gene in *P. vivax* populations circulating in endemic areas of Sri Lanka, where unstable malaria prevails with low transmission is extensively shown from the current study. Gunasekera *et al.* (2007) demonstrated that within the Sri Lankan population the signature of diversifying selection was detected most strongly in domain II of *Pvama-1*. The same was confirmed by Thakur *et al.* (2008b) from clinical isolates of India, although, Ord *et al.* (2008) revealed that the Venezuelan isolates showed a signifying diversity in domain I of *Pvama-1*. A lack of balancing selection contributing to the pattern of polymorphic substitutions in domains II or III was suggestive in both *P. vivax* and *P. falciparum* (Polley and Conway 2001; Gunasekera *et al.* 2007; Thakur *et al.* 2008b). Previous diversity studies on *P. vivax* were solely focused on domain I (Figtree *et al.* 2000; Rodrigues *et al.* 2005) though no evidence for diversifying selection was recorded, however in *P. falciparum* this was confirmed by previous investigations (Polley and Conway, 2001; Polley *et al.* 2003).

The present study focused on the genetic diversity of domain II in Sri Lankan field isolates taken from two endemic areas (EA), Anuradhapura and Kataragama, and a non-endemic area (NEA), Colombo. In order to draw comparisons on the genetic diversity of *Pvama-1* domain II within Sri Lanka, isolates from the two endemic areas (EA) were combined as the number of isolates representing each area was comparatively less than those from the non-endemic area (NEA). As previously mentioned, clinical isolates collected from residents of Colombo, were those who had travelled to malaria endemic areas in the island (Fonseka and Mendis, 1987). In addition, comparisons were made on domain II of *Pvama-1* sequences between local isolates with those previously published world wide isolates to study the genetic polymorphism and evolutionary relationships on a larger scale.

A plausible explanation for the high nucleotide diversity (π) and average number of nucleotide difference (k) evident among the NEA group may be that, the origin of these non-endemic isolates were traced back to 11 different administrative districts (Ampara, Anuradhapura, Hambantota, Jaffna, Kurunagala, Monaragala, Mullaitivu, Pollonnaruwa, Puttalum, Ratnapura and Vavuniya) of the island, 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 EA and the NEA, the difference between Dn-Ds was not

significant in the NEA group suggesting that the EA group had a tendency of stronger diversifying selection acting at PvAMA-1 domain II presumably to avoid host immune pressure, while the latter deviated from neutrality to a lesser extent. In the Tajima's D test, a negative value of D will imply an excess of alleles in low frequency, whereas a positive value will imply an excess of alleles in intermediate frequencies. However, an excess of alleles in intermediate frequencies is expected in a structured population, whereas an excess of alleles in low frequency is expected if the population is growing (Escalante *et al.* 2004). Therefore, the NEA group demonstrated purifying selection at 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 at PvAMA-1 domain II in the Sri Lankan patient population compared to the Indian population (Thakur *et al.*, 2008b) was further reiterated by the sliding window option where the nucleotide diversity ranged from 0.007-0.026 and 0.004-0.02 in Sri Lanka and India, respectively. Moreover, similar haplotype diversity results were obtained from Sri Lanka and India compared to Venezuela (Thakur *et al.* 2008b, Ord *et al.* 2008), which coincided with the number of amino acid haplotpes obtained from Sri Lanka (21 amino acid haplotypes), 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 supports the fact that sequence diversity with regard to 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 at *ama-1* is maintained by two evolutionary forces (Escalante *et al.* 2001); i) intragenic 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*. This was previously observed by Gunasekera *et al.* (2007) and Thakur *et al.* (2008b). However, the linkage disequilibrium graph 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 occurring at this gene in Sri Lanka. This was further reiterated by the 21 amino acid haplotypes generated due to 11 amino acid substitutions, which is discussed later on. Anderson *et al.* (2000) elucidated the observed association between transmission intensity and LD in *P. falciparum* by a mixed mating system in which inbreeding predominates in low transmission areas, while higher levels of out breeding occur in regions with higher transmission. This may be presumed since residents of low transmission regions are rarely super infected with more than one parasite clone during infection. As a result, unrelated parasites rarely co-occur in the same mosquito blood meal. Conversely, multiple clone infections are frequent where malaria transmission is intense. Consequently, mosquitoes frequently ingest unrelated parasites, leading to higher levels of out breeding (Babiker *et al.* 1994; Paul *et al.* 1995). In populations with high levels of inbreeding, the ''effective'' recombination rate will be considerably reduced. However, Anderson *et al.* (2000) highlighted a spectrum of population structures, with strong linkage disequilibrium and low genetic diversity in areas with low levels of transmission; and linkage ''equilibrium'' and high diversity in areas with high levels of transmission in *P. falciparum*.

The *ama-1DII* gene in Sri Lanka was diverse with the enclosure of single nucleotide polymorphisms (SNPs), where eleven amino acid (a.a) variants positions defined 21 a.a. haplotypes. Three haplotypes represented isolates common to all three test areas were those patients who had visited Anuradhapura, which is endemic to malaria prior to occurrence of the disease. Though, amino acid haplotype H1 being predominant in Sri Lanka and identical to the Sal I strain was observed in both Sri Lanka India, 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. Strikingly, an amino acid haplotype which was observed in an Indian isolate was also observed in a clinical isolate collected from a patient from Colombo who had no previous malaria infections and had visited India prior to acquiring 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 in amino acid haplotypes H8, H14, and H16 that consisted isolates common to Venezuela and Sri Lanka.

F_{ST} is a statistical value denoting the geographic difference between two populations, often expressed as the proportion of genetic diversity due to allelic frequency differences among populations (Holsinger and Weir, 2009). Genetic differentiation among populations has a

predictable relationship to the rates of important evolutionary processes (migration, mutation and drift). For example, large populations among which there is much migration tend to show little differentiation, whereas small populations with little migration tend to be highly differentiated. The fixation index values which indicated low genetic differentiation between Sri Lanka and India due to possible regular migration was reflected in the phylogenetic tree, where Sri Lankan isolates were mixed among Indian. A similar observation was marked between Venezuelan and Indian isolates where the genetic differentiation was low. Conversely, the intermediate genetic differentiation between Venezuela and Sri Lanka was clearly reflected in the phylogenetic tree, where those isolates from Sri Lanka, India and Venezuela that had common amino acid haplotypes were mixed together but was not clustered. Moreover, the phylogenetic tree constructed using all the domain II *ama-1* sequences from geographically different areas worldwide including Sri Lankan, revealed fewer clustering where most isolates had a very recent common origin and most of the changes were singletons. Although most branches in the phylogeny of domain II of *Pvama-1* sequences were not subjected to clustering, it was clear that some Venezuelan sequences clustered among each other but did not cluster separately from old world sequences of Sri Lanka and India. This pattern was consistent in the study by Grynberg *et al.* (2008), where the domain I *Pvama-1* sequences from Brazilian isolates belonging to the new world were found throughout the phylogenetic tree suggesting the hypothesis that the alleles established in the new world represented a sample of worldwide genetic diversity at the *Pvama-1* locus. There was evidence of reduced genetic diversity at the *Pvama-1* domain II locus in Venezuela, consistent with hypothesis that some reduction in effective population size of *P. vivax* occur on the new world after its introduction (Grynberg *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.

This particular geographical structure of diversity was suggestive of strong constraints acting on the evolution of AMA-1 at the population level (Cortes *et al.* 2003). The similarities observed in mutations between distant populations though with diverse amino acid haplotypes was likely to reflect the maintenance of similar balanced frequencies of the individual polymorphisms by the selection pressure of protective immune response. Further, different populations are expected to show substantially different allelic frequency distributions due to divergent population histories, including the effects of genetic drift. However, the significant clustering of isolates was restricted due to the single polymorphic substitutions occurring in this domain.

A 58.9% sequence identity was found when the sequence of PfAMA-1 from the FVO strain was modelled on the PvAMA-1 crystal structure (Chesne-Seck *et al.* 2005). Though sequence differences between these two species are distributed over the entire ectoplasmic region, domain I of PfAMA-1 was found to be more variable (Polley and Conway, 2001). Recent evidence from Venezuela (Ord *et al.* 2008), which demonstrated the diversity of both Pf and PvAMA-1 showed diversifying selection acting at domain I, further reiterating this fact. However, an investigation using Sri Lankan isolates was carried out to analyse the diversity of domain II of both Pf and PvAMA-1. Predominance of non-synonymous over synonymous mutations detected in both Pf and PvAMA-1 domain II implied that sequence diversity was maintained to counter the host's immune response (Polley and Conway, 2001; Gunasekera *et al.* 2007). Both amino acid polymorphic sites observed in PfAMA-1 (Chesne-Seck *et al.* 2005) and PvAMA-1 (Thakur *et al.* 2008b) was exposed on the surface. Moreover the distribution of these polymorphic residues in both species was highly biased to one side of the molecule. This suggested that this part of the region may be more exposed to the exterior on the parasite surface and or less susceptible to functional constraints. Presence of highly correlated sequences at particular amino acid polymorphic sites were present and these were well separated from each other in the tertiary structure, did not appear to be directly caused by structural factors in the protein itself but rather by genetic factors (Chesne-Seck *et al.* 2005). Furthermore, Ord *et al.*(2008) found genetic diversity to be significantly higher in the *P. vivax* population, and indices of intra-population recombination were significantly lower in the *P. falciparum* population, therefore concluding that whereas the *P. vivax* population comprise of a diverse gene pool that frequently undergoes recombination, the *P. falciparum* population exhibits restricted diversity and appears to propagate most commonly by clonal expansion, with minimal opportunities for recombination. This was further reiterated by the pairwise inter-population comparisons carried out for both *P. falciparum* and *P. vivax* AMA-1 domain II, where *P. falciparum* showed majority of diversity within each population than *P. vivax* (Ord *et al.* 2008).

In Sri Lankan isolates, PvAMA-1 domain II was subjected to positive selection, with frequent occurrences of non-synonymous substitutions relative to synonymous ones, presumably to avoid host immune pressure and signified that the parasites were under biological functional constraints as well as being targets of host immune mechanisms. Similarly, a positive selection was found to be acting on domain II of PvAMA-1 from 61 Indian isolates (Thakur *et al.* 2008b) as well as from 45 Venezuelan isolates collected in 1997 ($P>0.05$), while 28 isolates collected in 1997 from Venezuela were under purifying selection (Ord *et al.* 2008).

This indicated the tendency for stronger diversifying selection at this domain due to immune pressure from the host.

The McDonald Kreitman test for PvAMA-1 domain II of Sri Lankan patient population demonstrating a significant departure from neutrality with an excess of non-synonymous mutations within *P. vivax* sequences ($P<0.01$ based on Fisher's exact test) indicated purifying selection acting upon this domain, which operates to reduce the number of non-synonymous substitutions between species, *P. vivax* and *P. cynamolgi*. Though similar non-synonymous substitutions were present both within *P. vivax* and between species from India, Venezuelan isolates showed a departure from neutrality with an excess of non-synonymous mutations between species.

Domain II of PvAMA-1 is subjected to strong selection pressure in Sri Lanka. Every statistical test examined rejected the null hypothesis that the domain was neutral to selection. The direction of this selection, either positive towards greater diversity or negative towards elimination of mutations, varied depending on the type of statistical test used. The high rates of non-synonymous mutations relative to synonymous mutations on PvAMA-1 domain II indicated positive selection promoting greater diversity, presumably to avoid host immune responses. Moreover though not significant, a positive Tajima's test further reiterated this point. This was supported by the high degree of nucleotide diversity in the parasite population. Other statistical tests, such as the McDonald Kreitman test however indicated selection on domain II which was acting instead to reduce this diversity by elimination of non-beneficial mutations. However, it was suggested that the resulting observation could be due to a single *P. cynomolgi* sequence being used as the out group against 64 *P. vivax* sequences (Escalante, A. A. Personal communication). This indicated that different tests of neutrality (Dn-Ds, Tajima's test, McDonald Kreitman test) have a barrier on the outcome of results. However, the selection of neutrality using the difference of Dn and Ds can be strongly corroborate for the outcome of results at PvAMA-1 domain II in the clinical isolates of Sri Lanka than the use of the McDonald Kreitman test (Escalante, A. A. Personal communication).

During homology modelling, all the 21 amino acid haplotype sequences were modelled against the H1 haplotypes. Haplotypes H1 was the predominant haplotypes of PvAMA-1 domain II distributed in the *P. vivax* population in Sri Lanka and it was of great importance to perceive at the deviation from this haplotypes within the island due to the host immune

pressure. It was observed that all the haplotypes were 99.2-96.3% homologous to the domain II of PvAMA-1 Sal I strain. This was further reiterated by the majority of Sri Lankan isolates being distributed closely to the Sal I strain. Though, several amino acid haplotypes gained homology percentages common to them, the polymorphic amino acid substitution sites were distributed differently, giving rise to different protein sequences, which may eventually result in different antibody responses.

T and B cell epitopes were specifically predicted with respect to the four MHC alleles, which were frequently occurred in Africa and Asia as documented by Southwood *et al.* (1998). It was evident that regions of T cell epitopes overlap with B cell epitopes (Joshi *et al.* 2000; Escalante *et al.* 2001). Further 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). 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, where a existing epitope was absent while a new epitope was predicted due to polymorphism, to two MHC alleles, 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). The 4G2 B cell epitope discussed above overlapped with a highly immunogenic T helper cell epitope at PvAMA-1DII (Lal *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 residue length within the different *Plasmodium* orthologous but has lower sequence identity (21%) compared to the complete ectoplasmic region (39%). Nonetheless, no polymorphic sites have been detected to date in the domain II loop in *P. vivax*, suggesting that functional constraints may operate to prevent antigenic variation in this surface exposed segment (Chesne-Seck *et al,* 2005).

Polymorphism at this domain affected the B cell epitopes predicted, specially in the N terminal end of domain II. A dimorphic change that occurred from Lysine (K)/ Isoleucine (I) resulted in a decrease of the binding score may be as result of the polar non-polar change of the two amino acids, which may also be reflected in another polymorphic position where a change had occurred from Leucine (L) to Arginine(R). However, a dramatic decrease in the

binding score as a result of a polymorphic change from Glutamine (Q) to Arginine (R) cannot be explained by this hypothesis.

In conclusion, the present study demonstrated the diversifying nature of the *P. vivax ama-1* gene probably due to host immune pressure. The phylogenetic study revealed the relatedness of Sri Lankan patient's population 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. As discussed above, 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 and it demonstrated the present of a conformational epitope indicative of a functional importance to the protein. Finally, the amino acid sequence of the domain II loop will be an ideal vaccine candidate to be used in a combine subunit vaccine in the future.

CHAPTER 4

Genetic diversity of *Plasmodium vivax* Merozoite Surface Protein-1_{42} (*Pvmsp-1_{42}*) in Sri Lanka

4.1. Introduction

Acute clinical malaria which results in lysis of host erythrocytes is associated with repeated replication of the asexual blood stage parasites in circulating erythrocytes (Serrano *et al.* 2006). An effective vaccine against the erythrocytic stages of malaria parasites would be expected to limit parasite multiplication rates and thereby reduce morbidity, mortality and spreading of drug resistant parasites. 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 (Jośe *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, 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). Studies on Merozoite Surface Protein-1 (MSP-1) have been driven by the need to understand its role in parasite invasion of red blood cells, its importance as a target of protective immunity for the host and its possibility as a component of a malaria vaccine. These studies have highlighted the need for structural and functional analyses to provide an understanding of the importance of MSP-1. Much of the work on MSP-1 has been focused on the *P. falciparum* protein or that of other malaria parasite species used as models, but *P. vivax* is also of particular importance for vaccine development as it's becoming a burden in most tropical regions in the world (Babon *et al.* 2007).

4.1.1. Merozoite Surface Protein-1

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-1_{42} (Holder *et al.* 1992). During erythrocyte invasion secondary cleavage of MSP-1_{42} leads to shedding of the complex, that generates a 33 kDa fragment (MSP-1_{33}), derived from the N terminus of MSP-1_{42} (Blackman *et al.* 1991), while MSP-1_{19}, the second product of the cleavage remains on the surface of the parasite as it enters the red blood cell (Blackman *et al.* 1990).

4.1.2. Structure of C-terminal fragment of MSP-1

Molecular modeling of the three-dimensional structure of the MSP-1 C-terminal fragment of *P. vivax* could provide a better understanding of how molecular structure relates to biological activity (Serrano *et al.* 2006). For example, the structure could assist in the prediction of T and B cell epitopes, help in the identification of prominent antibody binding sites, and anticipate the effect of mutation on immunogenicity using a structure-based approach. To date, the crystal structures of the C-terminal domains of *P. cynomolgi* MSP-1 (Chitarra *et al.* 1999), *P knowlesi* MSP-1 (Garman *et al.* 2003), and the solution structure of *P. falciparum* MSP-1 (Morgan *et al.* 1999) have been reported.

Interactions between different regions of MSP-1 (Daly *et al.* 2001; Kauth *et al.* 2003) and between MSP-1, MSP-6 and MSP-7 (Mello *et al.* 2002; Kauth *et al.* 2006) or MSP-1 and MSP-9 also known as acidic–basic repeat antigen (ABRA) (Li *et al.* 2004; Kariuki *et al.* 2005) have been examined. Others have proposed regions of MSP-1 that binds to erythrocyte surface proteins (Nikodem and Davidson, 2000; Espinosa *et al.* 2003; Goel *et al.* 2003; Han *et al.* 2004).

The best structural information available for MSP-1_{19} is that it comprise of two epidermal growth factor EGF-like motifs that have a number of inter-domain contacts that are closely packed and are aligned anti parallel to one another (Miller *et al.* 1993; Takala *et al.* 2006; Babon *et al.* 2007). This results in a U-shaped conformation that brings the N- and C-termini into close proximity. As the C-terminus is directly bound to the merozoite membrane through a GPI anchor (Holder *et al.* 1994), this configuration means that both termini lie close to the membrane *in vivo*. The protein has a flat, disk-like shape with a highly charged surface. While MSP-1_{19} is part of the larger MSP-1_{42} precursor, it exists as an independent domain with no stable contacts to the rest of the sub-unit. Gel filtration and analytical ultracentrifugation experiments indicate that *P. vivax* MSP-1_{42} exists as a dimer in solution. MSP-1_{19} itself is a monomer, however, 35 amino-acids immediately upstream of its N-terminus are sufficient to cause dimerization (Babon *et al.* 2007). This signifies that if MSP-1_{42} exists as a dimer *in vivo*, secondary processing would cause the dissociation of two tightly linked MSP-1_{19} proteins on the merozoite surface just prior to invasion. Each EGF-like domain of MSP-1_{19} is held together primarily by a number of disulphide bonds, with the proportion of regular secondary structural elements being quite low. Domain II contains three disulphide bonds in classical EGF domain pattern between cysteines 1–3, 2–4 and 5–6. Both domains contain two anti-parallel beta strand pairs present in the *P. knowlesi* (Garman *et al.* 2003) and *P. cynomolgi* (Chitarra *et al.* 1999) MSP-1_{19} structures. Overall the *P. vivax* MSP-1_{19} structure is very similar to both the *P. knowlesi* and *P. cynomolgi* structures, where as the similarity to *P. falciparum* MSP-1_{19} is more limited (Morgan *et al.* 1999). The C-terminal region of MSP-1, which represents a conserved segment within this variable molecule, shows high sequence identity within *Plasmodium* species: *P. vivax* shares 84% sequence identity with *P. cynomolgi*, 81% with *P. knowlesi*, and 54% sequence identity with *P. falciparum* (Serrano *et al.* 2006).

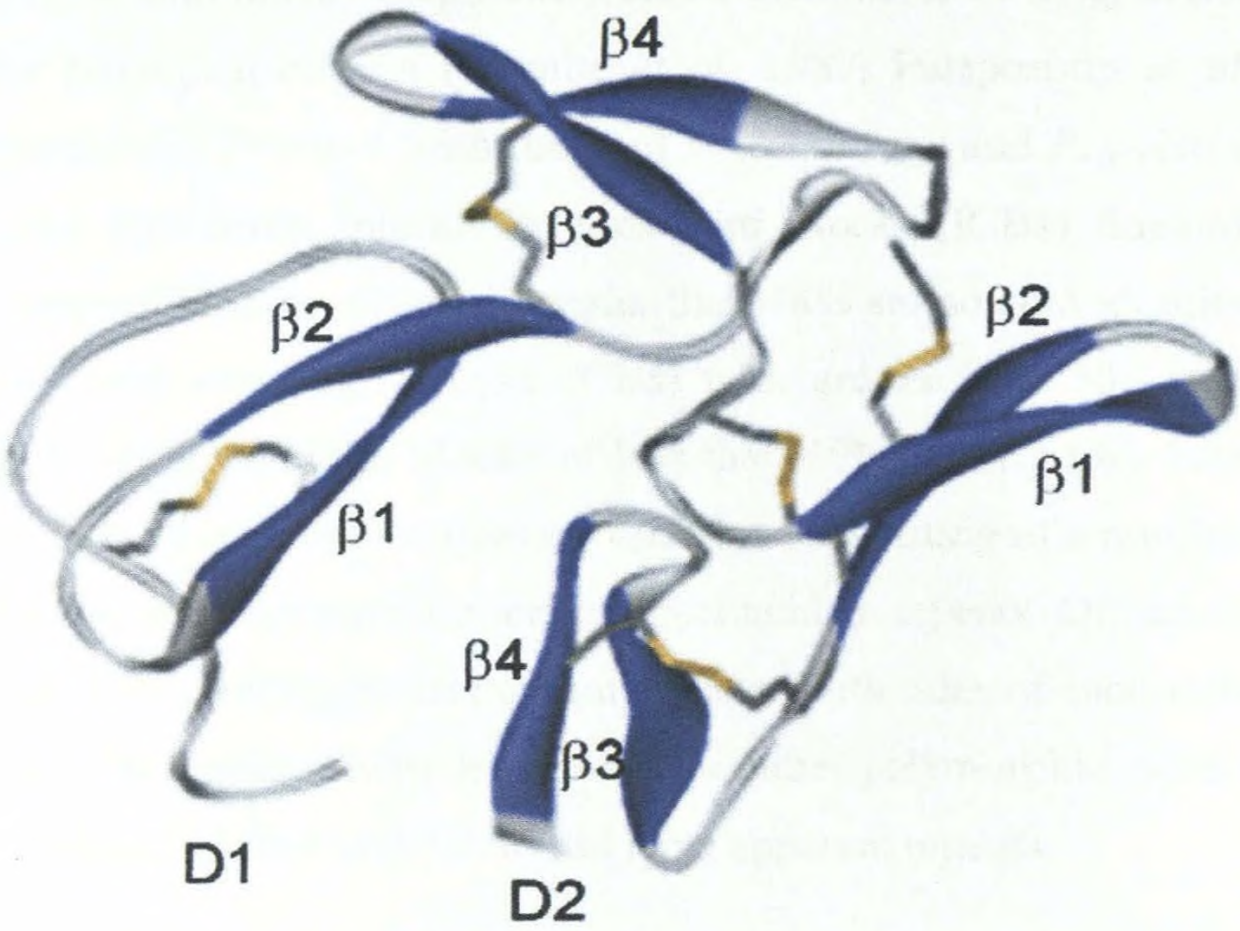


Figure 4.1. Ribbon representation of PvMSP-1_{19} including disulfide bonds, showing the backbone and the anti-parallel β sheet elements

(Adopted from Serrano *et al.* 2006)

In the case of *Plasmodium* species *msp-1*, most of the genetic diversity analyses has subdivided the gene into blocks (segments) based on their level of genetic diversity but not using any other biological criteria (Tanabe *et al.* 1987; Putaporntip *et al.* 2002 & 2006). Sequence comparison of *Pvmsp-1* with those of *P. falciparum* and *P. yoelii* demonstrated that it can be divided into seven interallele conserved blocks (ICBs) flanked by six variable blocks. The conserved blocks revealed greater than 48% amino acid identity among the three parasite species, three conserved blocks (CBs) with greater than 50% identity between *P. falciparum* and *P. vivax*, and nine blocks of less than 45% identity (del Portillo *et al.* 1991). Variable blocks showed extensive sequence variations consisting of a number of substitutions, insertions / deletions and varying numbers of short tandem repeats. Of the six variable blocks, blocks 2, 6, 8 and10 possess apparent tandem repeats. Both sides of each repeat are flanked by non-repeat unique sequences in which variation is rather polymorphic, with two to four basic sequence types, while blocks 4 and 12 do not have apparent repeats.

Alignments of *Pvmsp-1* alleles with those from four other *Plasmodium* species (described above) revealed that those amino acids that are perfectly conserved among all five species and those that are conserved in *P. vivax* and three of the other four species are distributed exclusively in conserved blocks. In all conserved blocks, the number of synonymous substitutions per synonymous site was greater than non-synonymous substitutions per non-synonymous site, with statistically significant differences in blocks 1, 3, 5, and 7, suggesting that structure-related functions are conserved in *msp-1* in nature, and therefore that purifying selection due to functional constraints is operating in conserved blocks (Putaporntip *et al.* 2002). Numerous recombination sites were distributed throughout *Pvmsp-1*, in both conserved blocks and variable block unique sequences, and the distribution was not uniform (Putaporntip *et al.* 2002).

To date several corrections to *msp-1* based on the protein sequence published by del Portillo, (1991) have been reported, some of these within the C-terminal 19 kDa fragment was reported by Putaporntip *et al.* (2002). Of the 91 residues, two were substituted, Methionin-1665 with Threonine in domain I, and Glycine 1684 with Alanine in the inter-domain linker region where the transition from domain I to domain II occurred (Serrano *et al.* 2006). Therefore, PvMSP-1_{19} fragment is neither of the Belem nor the Sal I origin.

Few studies have considered the proteolytic fragments as functional units (Escalante *et al.* 1998; Pacheco *et al.* 2007; Thakur *et al.* 2008a). The MSP-1_{42} and MSP-1_{19} fragments have

received special attention in *P. falciparum* as part of vaccine formulations given that they are relatively conserved and antibodies against these fragments inhibit the parasite invasion into the red blood cells (Yang *et al.* 1999; Stanisic *et al.* 2004). In addition, the critical role of the MSP-1_{19} fragment in the erythrocyte invasion is conserved even among distantly related species (O'Donnell *et al.* 2000).

4.1.3. Immuno-epidemiology of MSP-1

Studies on antibody responses against potential asexual erythrocytic *Plasmodium* vaccine candidates in endemic areas can help in understanding natural protective immune mechanisms that may provide useful insights for vaccine development. In particular, anti-merozoite antibodies have been shown to be active in *in vitro* functional assays, indicating a role in protective efficacy (Bouharoun-Tayoun *et al.* 1995; Kumaratilake & Ferrante, 2000). Antibody responses to C-terminal PfMSP-1 (MSP-1_{42} and MSP-1_{19}) are associated with a reduced risk of clinical malaria (Shai *et al.* 1995, Al-Yaman *et al.* 1996, Perraut *et al.* 2005). The prominence of IgG1 and IgG3 antibodies in the anti-PfMSP-1_{19} IgG response (Egan *et al.* 1995), and their association with clinical immunity has been described (Shi *et al.* 1996).

Comparatively little is known about PvMSP-1, due in part to the lack of an *in vitro* culture system for this parasite. However, molecular and processing homologies to PfMSP-1 suggest it has similar function(s) (Galinski & Barnwell, 1996), and may be involved in *P. vivax* merozoites binding to reticulocytes (Rodriguez *et al.* 2002). Importantly, baculovirus-expressed *Plasmodium cynomolgi* MSP-1 C-terminal 42 and 19 kDa recombinant proteins (Longacre, 1995; Holm *et al.* 1997) conferred a high degree of protection against a challenge infection in the toque monkey, *Macaca sinica* (Perera *et al.* 1998). These results are particularly notable because this natural host-parasite system is highly analogous to *P. vivax* infection in humans. Immuno-epidemiological studies have shown a high prevalence of antibodies against C-terminal regions of PvMSP-1 and an increase of IgG prevalence with exposure to *P. vivax* malaria in endemic regions of South America and Turkey (Soares *et al.* 1999; Zeyrek *et al.* 2008).

Serological studies have provided significant evidence to suggest that immune responses directed against the C-terminus MSP-1 (MSP-1_{19} and MSP-1_{42}) are associated with immunity in preclinical models (Long *et al.* 1994; Egan *et al.* 1999; Darko *et al.* 2005; Parkkinen *et al.* 2006) as well as from individuals residing in endemic areas (Egan *et al.* 1999; John *et al.*

2004). Wickramarachchi *et al.* (2007) demonstrated that, with increasing exposure to malaria in both endemic areas Anuradhapura and Kataragama of Sri Lanka, anti-PvMSP-1_{19} antibody responses were dominated by the functionally important IgG1 and IgG3 isotypes, with a concurrent reduction in IgM that was lacking in the non-endemic residents of Colombo. In contrast, the antibody switch for PvMSP-1_{42} was restricted to endemic residents with more extensive exposure. These results suggested that an IgM-dominated antibody response against the PvMSP-1_{42} polymorphic region in endemic residents may interfere with the development of an IgG-dominated ''protective'' isotype shift to PvMSP-1_{19}, that may complicate vaccine development.

While structural information is now considered indispensable for drug design, it is seldom considered necessary when designing vaccines. However, MSP-1_{19} highlights the importance of structural information in vaccine development. First, whilst it has been known for some time that antibodies against MSP-1_{19} can prevent parasite invasion *in vitro* (Blackman *et al.* 1990, O'Donnell *et al.* 2001) and can protect against malaria in rodent models (Valero *et al.* 1998), there is little evidence that total anti-MSP-1_{19} antibody levels correlate with protection against malaria in humans (Nwuba *et al.*2002). Second, whilst there are anti-MSP-1_{19} monoclonal antibodies that inhibit erythrocyte invasion (Blackman *et al.* 1990; Egan *et al.* 1999), there are others that are ineffective and even some that appear to block the action of inhibitory antibodies, the so-called "blocking" antibodies (Patino *et al.* 1997; Uthaipibull *et al.* 2001). Thus, the fine specificity of the anti-MSP-1_{19} antibody response may be a more reliable indicator of malarial immunity than total antibody titre (Nwuba *et al.* 2002; Corran *et al.* 2004). Recent studies have shown that invasion inhibitory antibodies bind to a specific region of *P. falciparum* MSP-1_{19} (Morgan *et al.* 2004 and 2005), therefore, structural information on the antigen provides valuable information for designing an improved antigen to elicit an effective immune response (Uthaipibull *et al.* 2001).

In the course of characterizing immune responses induced by MSP-1 vaccines, it was recognized that: (i) proteins produced by various expression systems differ in their immunogenicity and ability to induce anti-parasite activities (Arnot *et al.* 2008; Reed *et al.* 2009), (ii) not all MSP-1- based vaccines induce protective immunity (Stowers *et al.* 2001) and, (iii) the degree of inhibition was dependent on the method chosen to measure invasion- and growth inhibition mediated by anti-MSP-1_{42} and that only the methods that are based on cell viability and/or metabolic activity are able to accurately measure growth inhibition, while all the methods tested were equally able to detect invasion inhibition (Bergmann-Leitner *et*

al. 2006). Thus, selecting the appropriate method permits differentiation between the various mechanisms by which antibodies affect parasite development.

A study by Bergmann-Leitner *et al.* (2009) designed and investigated the effect of anti-MSP-1_{42} antibodies on intra-erythrocytic parasite development and their consequences on parasite viability and infectivity. Depending on the parasite clone, anti-MSP-1_{42} antibodies prevented schizont rupturing by stalling or arresting intra-erythrocytic parasite development, likely through direct interactions with intra-erythrocytic parasites within the parasitophorous vacuole. The nature of the inhibitory effect was strongly affected not only by the parasite clone, but also by the fine-specificity of the antibodies. Only antibodies that bound to the MSP-1_{19} subunit, but not the EGF-like domain I or II, displayed growth inhibitory activities indicating that protective epitopes are dependent on the tertiary structure of the molecule.

4.1.4. Genetic Diversity of *msp-1*

Distantly related in phylogeny, with a divergence time of 75 – 165 million years (Myr) ago (Escalante *et al.* 1995), *P. vivax* and *P. falciparum* greatly predate the origin of hominids. *P. vivax* and the related monkey malaria parasite species *P. cynomogi* and *P. knowlesi* diverged 11.7 – 30.5 Myr ago, whereas *P. falciparum* and the closely related chimpanzee malaria parasite *P. reichenowi* diverged 5 – 8 Myr ago (Escalante *et al.* 1995). Also, the genomic compositions of these species differ greatly where the A+T content in the protein-coding region is ≈75% in *P. falciparum* / *P. reichenowi* and ≈60% in *P. vivax* / *P. cynomolgi* / *P. knowlesi*. These facts suggest that the evolutionary history of polymorphism of *msp-1* differs greatly between *P. falciparum* and *P. vivax*. 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.* 2008a). 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-1$_{19}$ region, MSP-1$_{33}$ 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.* 2008a) 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, they 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.* 2008a) 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 *Anopheline* 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).

4.1.5. Objective of the current 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-1_{42} 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.

4.2. Research Design

The C-terminal fragment of the *P. vivax msp-1* gene was amplified using 95 *P. vivax* isolates from two malaria endemic areas (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 were:

1. To explore the level of diversity of the entire *Pvmsp-1*$_{42}$ gene as well as of the *Pvmsp-1*$_{33}$ and *Pvmsp-1*$_{19}$ 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 among other geographically different countries to determine the level of diversity prevalent in geographically diverse *P. vivax* populations.

3. To identify amino acid haplotypes within the local population and to compare its polymorphism between the two reported complete sequences of PvMSP-1$_{42}$ (the Salvador I and the Belem strains) and to compare them with other previously reported data.

4. To construct a phylogenetic tree based on *Pvmsp-1*$_{42}$ nucleotide sequences obtained from the local parasite isolates as well as other reported geographically diverse isolates to examine the evolutionary relationships of the present antigen.

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-1$_{33}$.

4.3. Results

DNA sequences coding for C-terminal 42 kDa *Pvmsp-1* (amino acid positions 1384–1722 of the Salvador I strain) were analysed from 95 Sri Lankan *P. vivax* field isolates giving readable PCR products of 1016 bp (of the 167 tested), from non-endemic Colombo (N=37) and endemic Anuradhapura (N=22) and Kataragama (N=36).

Analyses of genetic diversity for the 95 sequences were performed for the *Pvmsp-1*$_{42}$, *Pvmsp-1*$_{33}$ and *Pvmsp-1*$_{19}$ fragments in all three study areas and to the entire population.

4.3.1. *Pvmsp-1* sequence polymorphism for the three test areas

P. vivax msp-1$_{42}$ in entirety observed 57 polymorphic sites from Anuradhapura and 58 each from Kataragama and Colombo giving rise to 62, 63, and 64 point mutations, respectively. Neither the number of polymorphic sites nor mutations were significantly different among the three study areas (Chi square test, $P>0.05$). 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, while 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). Table 4.1, recorded a high nucleotide diversity (π) for both 42 ($\pi = 0.0238$) and 33 kDa ($\pi = 0.0319$) fragments from Kataragama, and a low π for 19 kDa fragment from Anuradhapura (Table 4.1)

Differences between the nucleotide diversity of non-synonymous mutations per non-synonymous site (Dn) and that of synonymous mutations per synonymous site (Ds) were indicative of positive selection acting on the 42 kDa fragment from Anuradhapura (Dn-Ds = 0.0108, $P<0.05$), and the 33 kDa fragment from both Anuradhapura and Colombo (Dn-Ds = 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 (Dn = Ds) was rejected in favour of the alternative hypothesis (Dn > Ds). 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). Taking *P. cynomolgi* as the out group, the McDonald-Kreitman test for neutrality was applied to the entire PvMSP-1$_{42}$, PvMSP-1$_{33}$ and PvMSP-1$_{19}$

within the three test areas (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. Particularly, Anuradhapura showed a significant departure from neutrality (NI=2.062, P<0.001) compared to Kataragama (NI=2.019) and Colombo (NI=2.042) and was evident for strong positive selection at PvMSP-1_{42}. Surprisingly PvMSP-1_{33} 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 (Rm) observed in *Pvmsp-1_{33}*, 11 were common to all three study sites, while a single minimum recombination event (Rm) was detected between nucleotide positions 4941 and 4959 in a single isolate from Colombo (Table 4.3).

Comparison of the genetic differentiation within Sri Lankan *Pvmsp-1_{42}* diversity showed low F_{ST} values of –0.0205 between Anuradhapura / Kataragama, -0.0196 Anuradhapura / Colombo and -0.0177 Kataragama / Colombo; negative F_{ST} values can be interpreted as "0" and therefore no genetic differentiation was observed.

4.3.2. *Pvmsp-1* sequence polymorphism in Sri Lankan isolates

4.3.2.1. Genetic diversity at *Pvmsp-1_{42}*

Ninety five clinical isolates at the entire *Pvmsp-1_{42}* generated 58 polymorphic sites with 64 point mutations. All these polymorphic sites were parsimony informative sites (sites that have a minimum of two nucleotides that are present at least twice), with 53 having two, 4 having three, and a single site having four (nucleotide position 4550) variants. The sliding window analysis of DnaSP (window length 100 bp, step size 25 bp) revealed nucleotide diversity ranging from 0 to 0.15 (Figure 4.2). Most of the variations were concentrated from nucleotide positions 4350-4600 bp which is the central part of *Pvmsp-1_{42}*.

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

	π (S.D)	Ds	Dn	Dn-Ds	(Z-test)	Tajima's D Test
***Pvmsp-1*$_{42}$**						
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)
***Pvmsp-1*$_{33}$**						
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)
***Pvmsp-1*$_{19}$**						
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* of *P. vivax* and *P. cynomolgi*

Region	Interspecific fixed differences	Intraspecific fixed difference		Neutrality index[1]	Fisher's Exact Test[2]
		P. vivax (N=95)	*P. cynamolgi* (N=10)	M K test	(2- tailed)
Entire Population					
p42 Synonymous	49	8	42	2.042	0.010277*
Non-synonymous	46	43	57		
p33 Synonymous	38	6	35	2.219	0.010240*
Non-synonymous	36	43	47		
p19 Synonymous	11	2	7	1.222	1.000000
Non-synoymous	10	-	10		
Anuradhapura					
p42 Synonymous	49	7	42	2.062	0.009958**
Non-synonymous	46	42	57		
p33 Synonymous	38	6	35	2.192	0.0105*
Non-synonymous	36	42	47		
p19 Synonymous	11	1	7	1.375	0.7512
Non-synoymous	10	-	10		
Kataragama					
p42 Synonymous	49	7	42	2.019	0.0105*
Non-synonymous	46	43	57		
p33 Synonymous	38	5	35	2.192	0.0105*
Non-synonymous	36	43	47		
p19 Synonymous	11	2	7	1.222	1.000000
Non-synoymous	10	-	10		
Colombo					
p42 Synonymous	49	7	42	2.042	0.010277*
Non-synonymous	46	44	57		
p33 Synonymous	38	5	35	2.219	0.010240*
Non-synonymous	36	44	47		
p19 Synonymous	11	2	7	1.222	1.000000
Non-synoymous	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. * $0.01<P<0.05$; ** $0.001<P<0.01$;*** $P<0.001$

Table 4.3. Recombination sites for *Pvmsp-1*$_{33}$ and *Pvmsp-1*$_{19}$ among clinical isolates of Sri Lanka

Region	Study Site	R_m [a]	Recombination detected sites (with reference to the *Pvmsp-1* Salvador I sequence)
Pvmsp-1$_{33}$ (4510-4920)	Total Population (N = 95)	17	(4327,4360) (4360,4372) (4372,4426) (4445,4468) (4480,4525), (4525,4537) (4537,4544) (4544,4547) (4547,4548) (4549,4555), (4555,4558) (4558,4559) (4561,4570) (4581,4614) (4614,4692) (4692,4697) (4757,4784)
	Anuradhapura (N = 22)	13	(4360,4445) (4445,4468) (4480,4525) (4525,4537) (4537,4544), (4544,4547) (4547,4548) (4549,4555) (4555,4558) (4558,4559), (4561,4577) (4581,4614) (4614,4692)
	Kataragama (N = 36)	14	(4327,4360) (4372,4445) (4445,4468) (4480,4525) (4525,4537), (4537,4544) (4544,4548) (4549,4555) (4555,4558) (4558,4559), (4561,4577) (4581,4614) (4614,4692) (4757,4784)
	Colombo (N - 37)	13	(4360,4426) (4445,4468) (4480,4525) (4525,4537) (4537,4544), (4544,4548) (4549,4555) (4555,4558) (4558,4559) (4561,4570), (4581,4614) (4614,4692) (4692, 4697)
Pvmsp-1$_{19}$ (4921 - 5166)	Total Population (N = 95)	1	(4941, 4959)
	Anuradhapura (N = 22)	0	
	Kataragama (N = 36)	0	
	Colombo (N - 37)	1	(4941, 4959)

[a] – Minimum recombination events

Sites marked in red represents the common recombination detected sites occurring in all three study areas of Sri Lanka

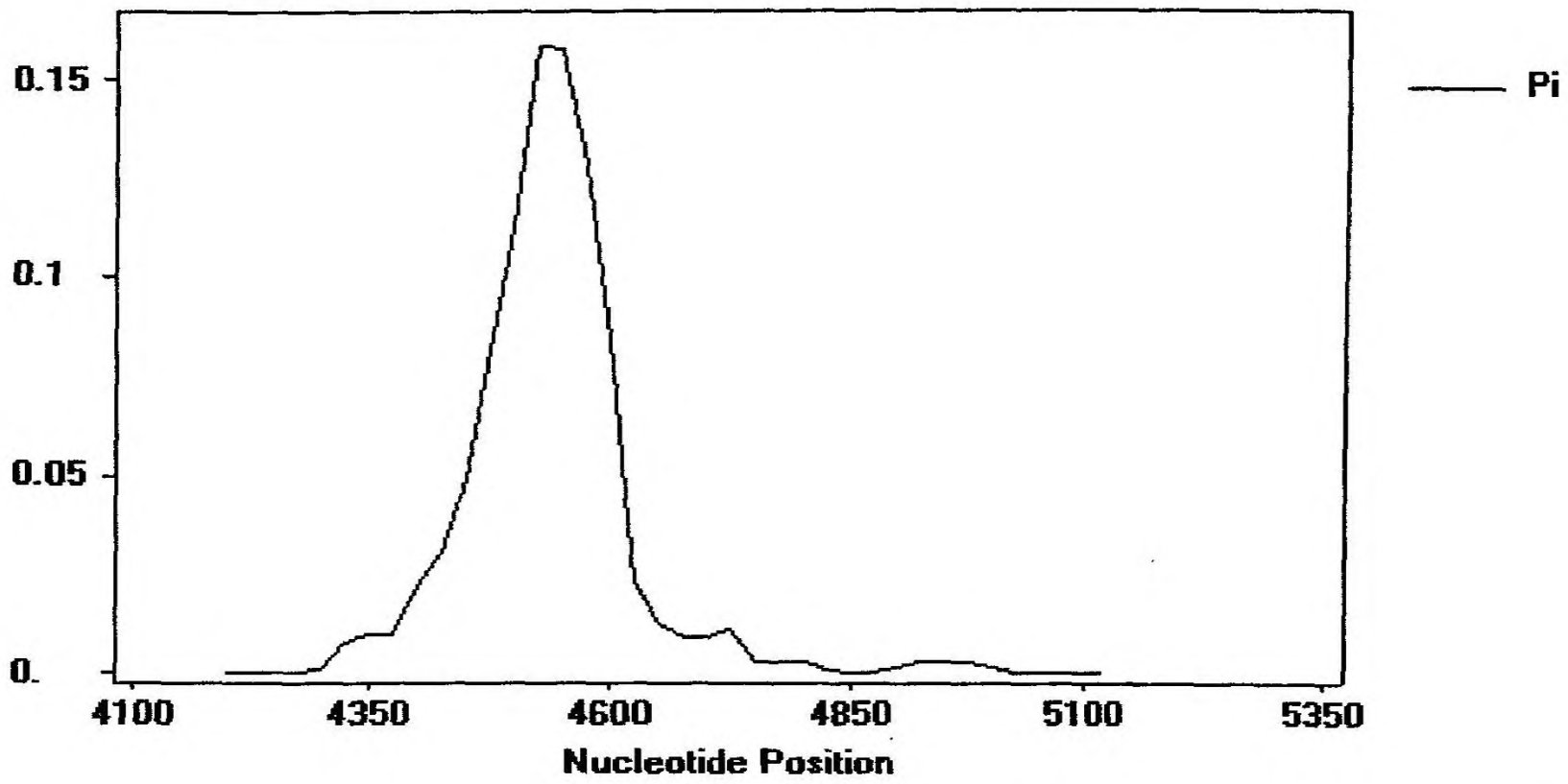


Figure 4.2. Sliding window plot of nucleotide diversity per site (π) comparing the level of genetic diversity at *Pvmsp-1*$_{42}$

The π values were calculated on DnaSP with window length 100 (bp) and step size of 25 (bp). Nucleotide positions 4150 – 5166 correspond to *Pvmsp-1*$_{42}$ of the Sal-I strain.

The nucleotide and haplotype diversities were highest from the population of India (N = 33), Hd = 0.977± 0.014, π = 0.0233± 0.0006, followed by Sri Lanka (N = 95), Hd = 0.962 ± 0.007, π = 0.0231 ± 0.0005; Thailand (N = 35), Hd = 0. 0.953 ± 0.024, π = 0.022± 0.001; Brazil (N = 11), Hd = 0.945 ± 0.054, π = 0.0221 ± 0.003; Bangladesh (N = 5), Hd = 0.7 ± 0.218, π = 0.019 ± 0.006 and South Korea (N = 4), Hd = 0.5 ± 0.265, π = 0.0005 ± 0.0003 (Putaporntip *et al.* 2000 and 2002; Thakur *et al.* 2008a). While a positive selection was documented at PvMSP-1_{42} by both Dn-Ds and the Tajima's D test, only the latter was significant (Table 4.1). Moreover, 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 by the McDonald-Kreitman test (Table 4.2).

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 *Pvmsp-1*$_{42}$ (Figure 4.3).

Comparing the F_{ST} value of *Pvmsp-1*$_{42}$ 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).

4.3.2.2. Genetic Diversity at *Pvmsp-1*$_{33}$ and *Pvmsp-1*$_{19}$

Fifty six polymorphic sites gave rise to 62 point mutations, at *Pvmsp-1*$_{33}$, where the highest nucleotide diversity was recorded compared to *Pvmsp-1*$_{42}$ and *Pvmsp-1*$_{19}$ (Table 4.1). Particularly, a polymorphic region of 113 bp at positions 4468-4581, having a clear excess of non-synonymous versus synonymous substitutions, confirmed previous reports as were overall π values at *Pvmsp-1*$_{33}$ in Table 4.1. (Manamperi, 2002; Pacheco *et al.* 2007; Thakur *et al.* 2008a). While, the difference between the non-synonymous mutations compared to synonymous mutations (Dn–Ds = 0.0134, P<0.01) and the Tajima's D test (2.97050, P<0.01) demonstrated a highly positive selection at PvMSP-1_{33}, the McDonald Kreitman test recorded contrary (Table 4.1 & 4.2).

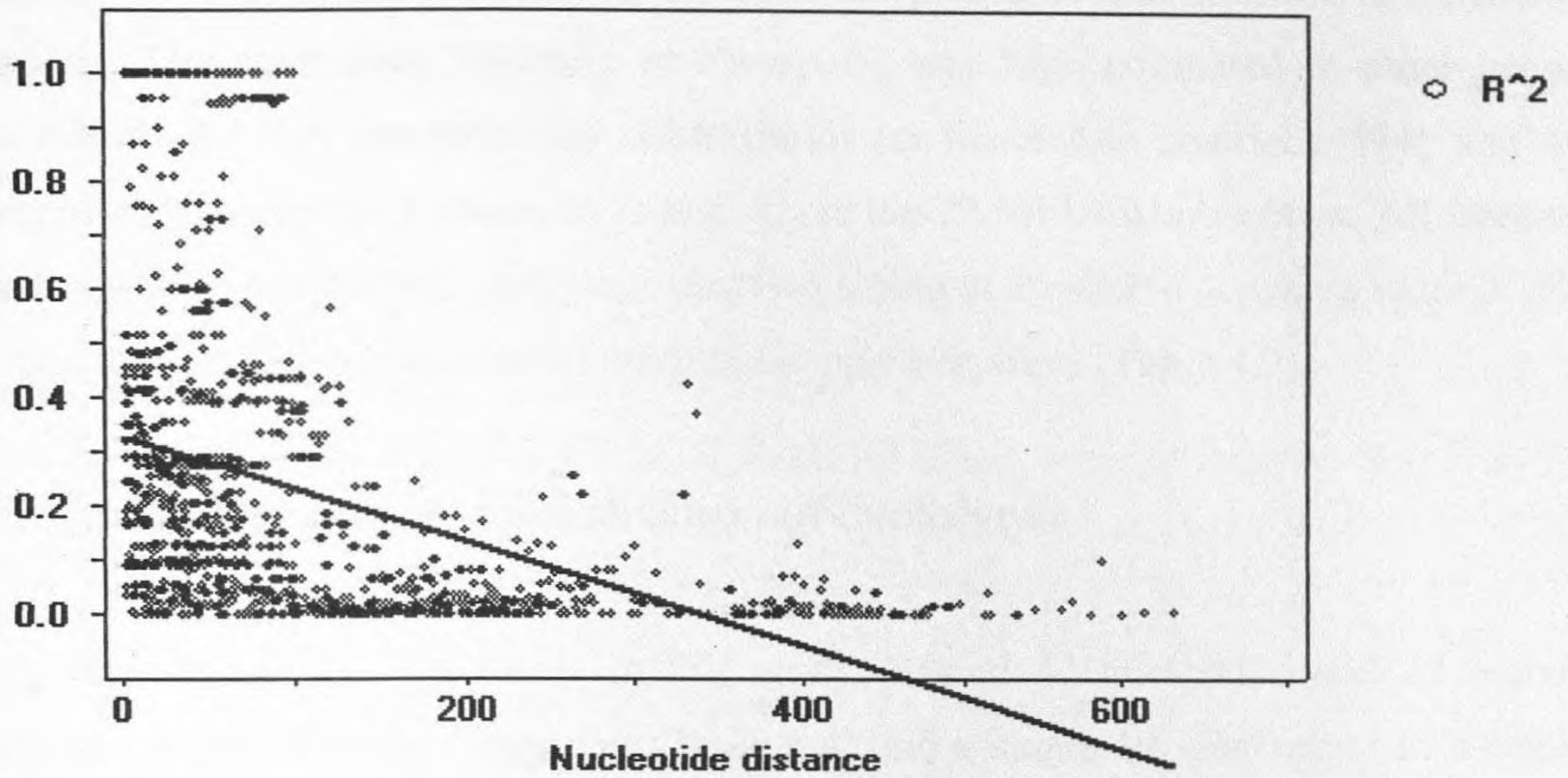


Figure 4.3. The linkage disequilibrium (LD) plot for the *Pvmsp-1*$_{42}$ region showing non-random association between nucleotide variants at different polymorphic sites

The R^2 values are plotted against the nucleotide distances with two-tailed Fisher's exact test of significance.

Conversely, *Pvmsp-1*$_{19}$ generated only two polymorphic sites that gave rise to 2 synonymous mutations. The nucleotide diversity at *Pvmsp-1*$_{19}$ was high compared to other geographic areas due to the two synonymous substitutions (at nucleotide positions 4941 and 4959bp corresponding to the Sal I strain) at *Pvmsp-1*$_{19}$ in the 12 Sri Lankan isolates. All three tests of natural selection confirmed purifying selection acting at PvMSP-1$_{19}$, where natural selection does not favour the maintenance of amino acid polymorphism (Table 4.2).

4.3.3. Classification and identification of haplotypes

Ninety five *Pvmsp-1*$_{42}$ sequences in this study defined 37 nucleotide and 27 amino acid haplotypes in the 33 kDa fragment (Table 4.4) and a single 19 kDa amino acid haplotype, generally confirming previous results (Putaporntip *et al.* 2000 & 2002; Manamperi, 2002; Pacheco *et al.* 2007). The 27 amino acid haplotype numbering were assigned in an increasing homology to the *Pvmsp-1*$_{42}$ Salvador I strain. 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). Amino acid haplotype H7 found to be predominant in Sri Lanka were reported by 11 of the 95 field isolates (11.6%). Fourteen of the 27 amino aicd haplotypes were common to Colombo and to those from the two endemic areas (Table 4.5). Published 202 *Pvmsp-1*$_{42}$ 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: accordingly, N-terminal region I (RI) upstream of the HVR block with 7 variant positions, a central 38 amino acid hypervariable region (HVR or RII) including positions 1490-1527 with 29 variants, and region III (RIII) downstream of the HVR block and upstream of the 19 kDa fragment with 3 variant positions were defined for this study (Table 4.4.). All variant positions in RI and RIII and over half (19 of 29 variant positions) in RII (HVR) were dimorphic, with only two alternating amino acid residues observed in isolates from this and all other studies cited (Table 4.4.).

RI of the present Sri Lankan cohort included 5 more variant positions compared to Manamperi's (Manamperi, 2002) Sri Lankan cohort, but none of those RI haplotypes was 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 4.4. and 4.5.), 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 (Tables 3). Four haplotypes from other studies (H70, 76, 77, 75) defined an eighth variant position in RI (1468-L>V; Table 4.4).

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 4.4 and 4.5).

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 4.4. and 4.5.). 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 4.4. and 4.5.). 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 4.4. and 4.5.). 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 4.4. and 4.5.). 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, defined the 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, 2002), two from India and one from South Korea, respectively, while HVR-T2 (Sal I, H62–H70) comprised the majority global isolates (N=17). Surprisingly, HVR-T3 was identified in

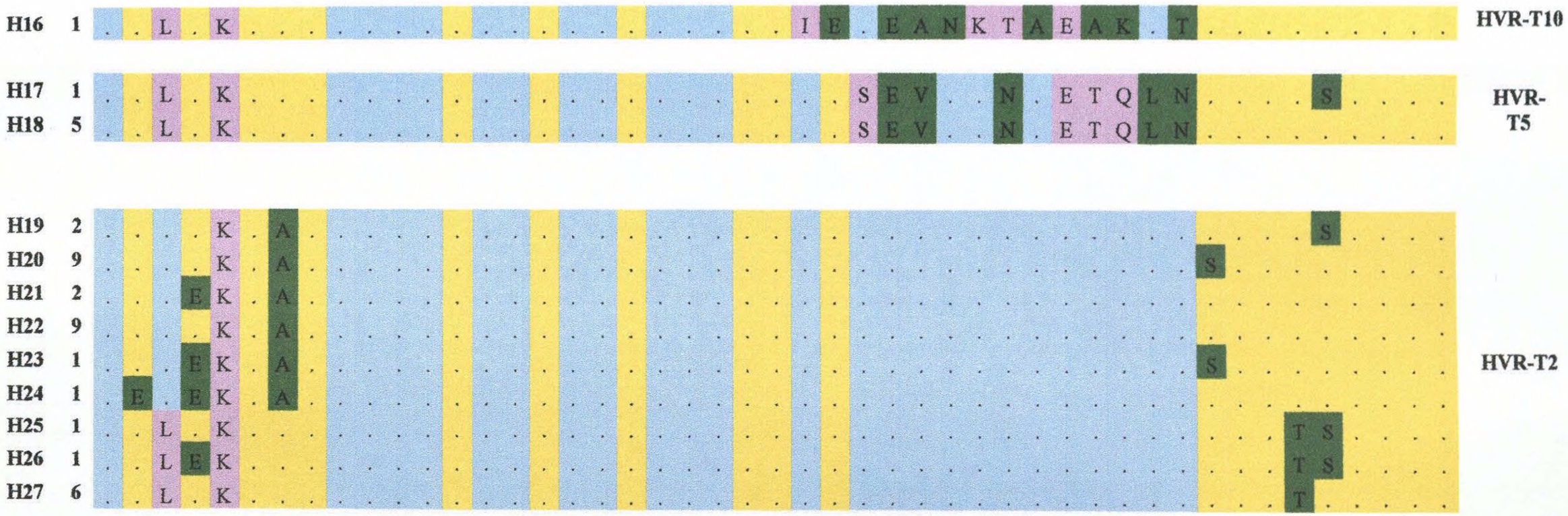
only three other isolates (H41-H43 from Sri Lanka, India and Thailand, respectively). HVR-T4 recurred in H51-H57 and included two isolates each from Thailand and Brazil, one each from Bangladesh, Japan, Sumatra and Vanuatu. HVR-T5 was relatively rare, and included only two isolates from India (H58 and H59) (Table 4.4.). Though, HVR-T6 was seen in H31 and H32 from India (one isolate each) and H33 from Bangladesh (three isolates), HVR-T7 was not observed in any global 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 4.4.). Finally, two new HVR types T11 and T12 representing a double recombination between HVR-T2/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) with 21 global isolates lacked interallelic double recombination, rather they were generated solely by intragenic recombination (Table 4.4.).

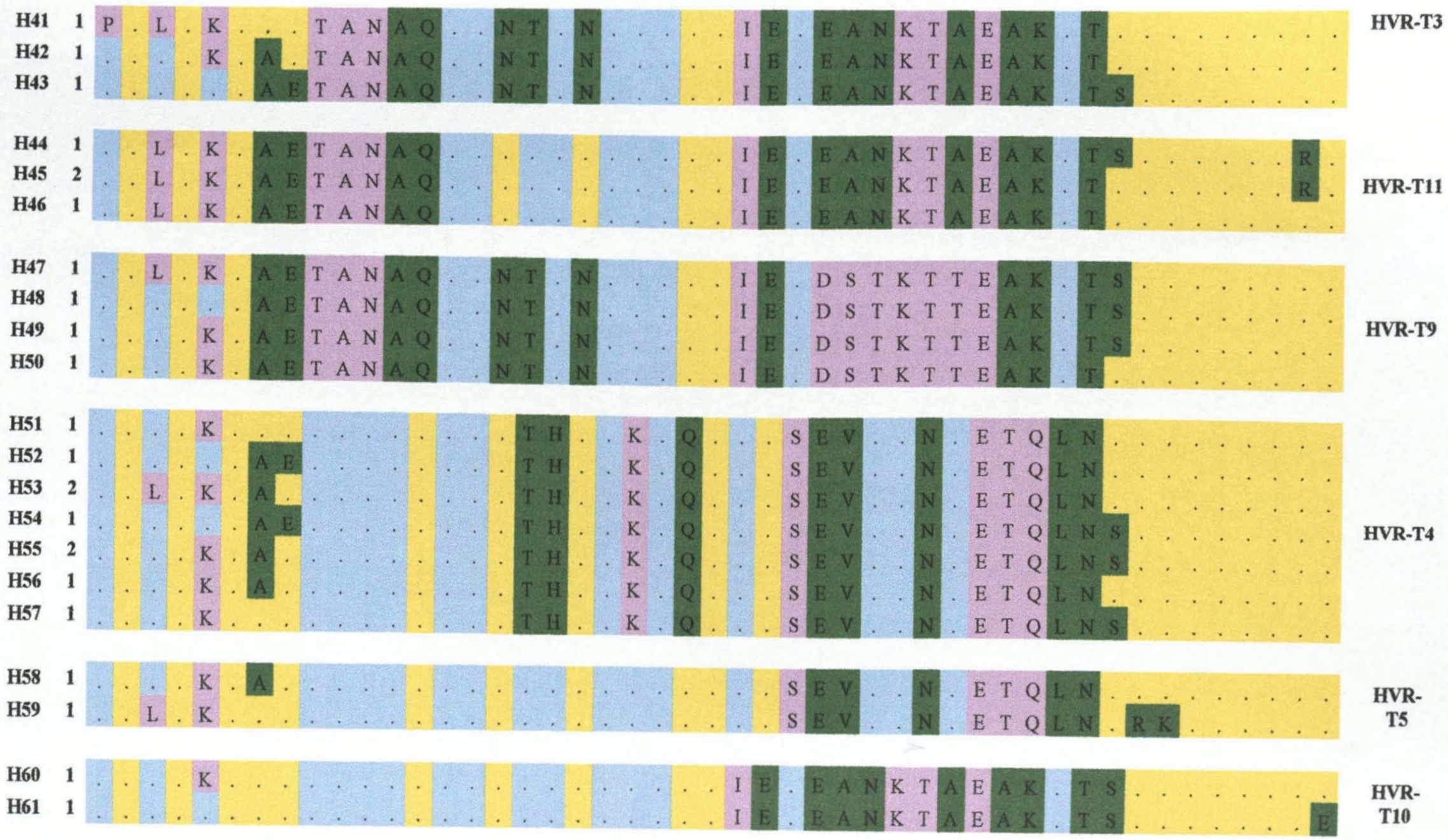
Altogether, 202 sequences defined 89 amino acid haplotypes (Table 4.4.), of which eight PvMSP-1_{42} haplotypes H2, H5, H6, H7, H9, H12, H14 and H18 from this study were found in other global isolates representing HVR types T1, T3-T6 and T8-T9. While most of the haplotypes were observed in India (H5-02, 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, 2002) 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-1_{42} 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 4.4). 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 K/E-1458 were observed (Table 4.4.). Dimorphic sites at positions 1573, 1574 were observed in a single isolate from Thailand, while 1597, 1601 was observed in an Indian isolate (Table 4.4.).

Table 4.4. Amino acid polymorphism in the *P. vivax* MSP-1_{33} among geographically diverse malaria endemic areas

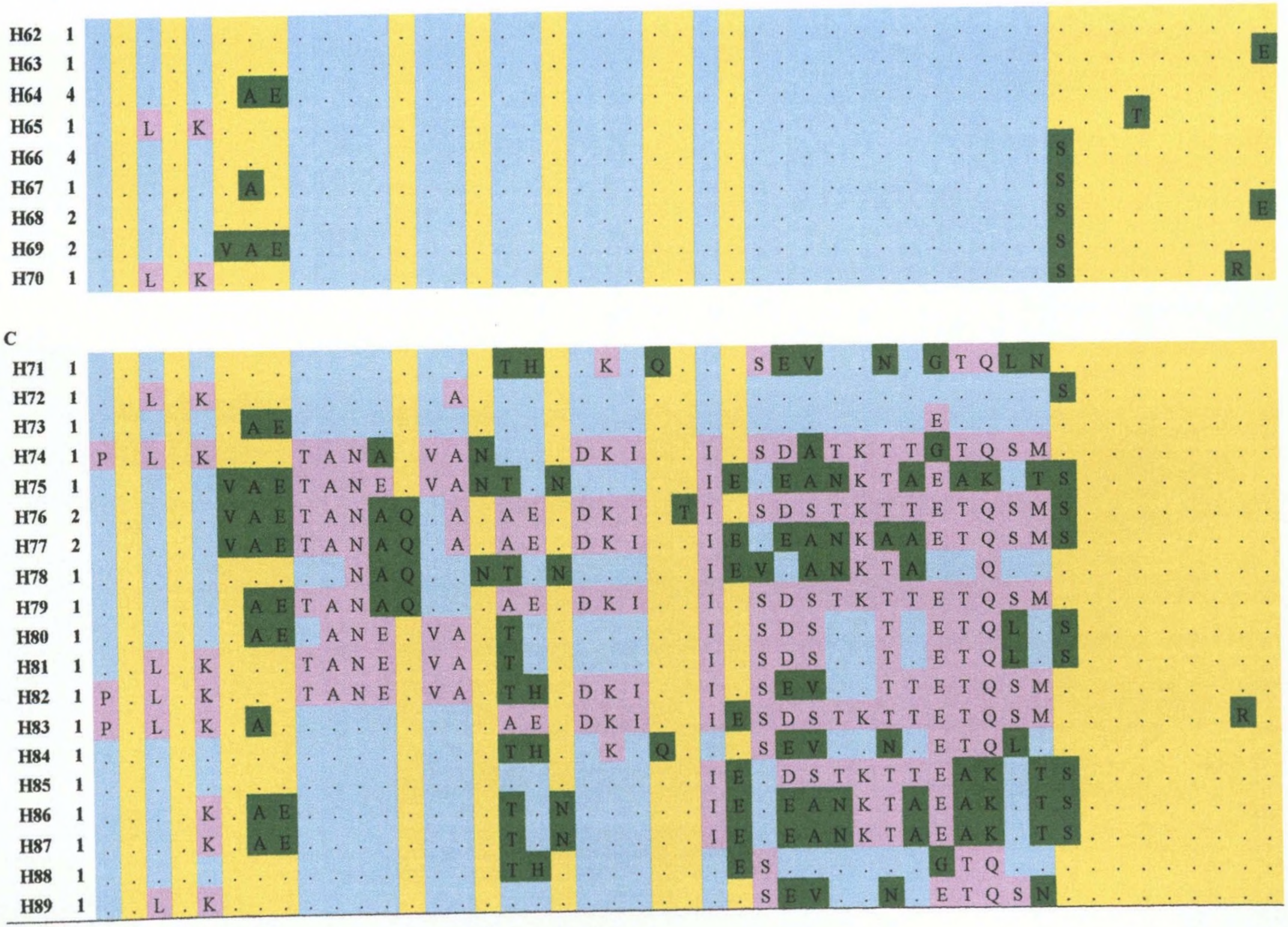
		1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1		
		4 4 4 4 4 4 4 4 4 4 4 4 4 4 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 6 6 7		
		3 4 5 5 6 6 7 8 9 9 9 9 9 9 0 0 0 0 0 0 0 1 1 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 6 7 7 8 9 9 0 1 0		
		2 3 4 8 6 8 6 2 0 1 3 4 5 6 1 4 5 6 7 8 9 0 1 2 3 4 5 6 7 8 9 0 1 2 4 5 6 7 6 3 4 6 5 7 1 2 9		
Belem*		P K L K K L T G T A N E E V A K A E D D K I K K I G S D S T K T T E T Q S M N K E N Y K K K K		**HVR**
Sal I#		S K I K M L T G D G K T E I D K K Q D E E L K K L G N V N S Q D S K N E F I N K E N Y K K K K		**types**
Amino acid haplotypes				
A				
	N			
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 . . . . S		
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 S		
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**


H16 1 I E E A N K T A E A K T HVR-T10
H17 1 S E V N E T Q L N S HVR-T5
H18 5 S E V N E T Q L N
H19 2 A S
H20 9 A S
H21 2 E A S
H22 9 A S
H23 1 E A A S HVR-T2
H24 1 E E A
H25 1 T S
H26 1 T S
H27 6 T
B
H28 2 P T A N E V A A E D K I I S D S T K T T E T Q S M
H29 2 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 S HVR-T1
H30 1 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
H31 1 A E 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
H32 1 A E 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 N T HVR-T6
H33 3 A 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 S
H34 7 A A E D K I E I S D S T K T T E T Q S M S
H35 1 A E A A E D K I E I S D S T K T T E T Q S M S HVR-T12
H36 3 A T A N E V A A E D K I I E E A N K A A E T Q S M S
H37 2 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
H38 1 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 S HVR-T8
H39 1 A T A N E V A A E D K I I E E A N K A A E T Q S M S
H40 1 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 S E


H41 1 P . L . K . . . T A N A Q . . N T . N . . . . . I E . E A N K T A E A K . T
H42 1 . . . . K . A . T A N A Q . . N T . N . . . . . I E . E A N K T A E A K . T
H43 1 . . . . . A E T A N A Q . . N T . N . . . . . I E . E A N K T A E A K . T S
HVR-T3
H44 1 . . L . K . A E T A N A Q . . . . . I E . E A N K T A E A K . T S . . . . . R .
H45 2 . . L . K . A E T A N A Q . . . . . I E . E A N K T A E A K . T . . . . . R .
H46 1 . . L . K . A E T A N A Q . . . . . I E . E A N K T A E A K . T
HVR-T11
H47 1 . . L . K . A E T A N A Q . . N T . N . . . . . I E . D S T K T T E A K . T S
H48 1 . . . . . A E T A N A Q . . N T . N . . . . . I E . D S T K T T E A K . T S
H49 1 . . . . K . A E T A N A Q . . N T . N . . . . . I E . D S T K T T E A K . T S
H50 1 . . . . K . A E T A N A Q . . N T . N . . . . . I E . D S T K T T E A K . T
HVR-T9
H51 1 . . . . K . . . . . T H . . K . Q . . . S E V . . N . E T Q L N
H52 1 . . . . . A E . . . . . T H . . K . Q . . . S E V . . N . E T Q L N
H53 2 . . L . K . A . . . . . T H . . K . Q . . . S E V . . N . E T Q L N
H54 1 . . . . . A E . . . . . T H . . K . Q . . . S E V . . N . E T Q L N S
H55 2 . . . . K . A . . . . . T H . . K . Q . . . S E V . . N . E T Q L N S
H56 1 . . . . K . A . . . . . T H . . K . Q . . . S E V . . N . E T Q L N
H57 1 . . . . K . . . . . T H . . K . Q . . . S E V . . N . E T Q L N S
HVR-T4
H58 1 . . . . K . A . . . . . S E V . . N . E T Q L N
H59 1 . . L . K . . . . . S E V . . N . E T Q L N . R K
HVR-T5
H60 1 . . . . K . . . . . I E . E A N K T A E A K . T S
H61 1 . . . . . I E . E A N K T A E A K . T S . . . . . E
HVR-T10

HVR-T2

C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H63 1
H64 4
H65 1
H66 4
H67 1
H68 2
H69 2
H70 1
H71 1
H72 1
H73 1
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H75 1
H76 2
H77 2
H78 1
H79 1
H80 1
H81 1
H82 1
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H85 1
H86 1
H87 1
H88 1
H89 1

Note: Amino acid variant positions 1432 – 1709 corresponded to the two reference strains of PvMSP-1_{33} 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 toT12 represents the PvMSP-1_{33} 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.

Haplotypes H2 were observed in 3 other South Korean isolates (AF435635, AF435636 and AF435638)

Haplotypes H5 were observed in other Indian isolates (EU430454, EU430454-2) H6 (EU430466), H9 (EU430457, EU430457-2), H12 (EU430470), H14 (EU430468, EU430471), H18 (EU430456, EU430456-2).

Haplotype H7 was observed in an Indian (EU430469) and 2 Brazilian isolates (AF435626 and AF435631).

Remaining haplotypes were from;

i) Sri Lanka (Manamperi, 2002) (H28- AJ292349 and AJ292352; H41- AJ292350; H58- AJ292358; H63- AJ292354; H64- AJ292359; H74- AJ292353; H78- AJ292357; H82- AJ292351; H84- AJ292355; H88- AJ292356;)

ii) India (Thakur *et al.* 2008a) (H29- EU430452, EU430452-2; H31- EU430477; H32- EU430479; H37- EU430453, EU430453-2; H38- EU430476, Thailand AF435622; H42- EU430458; H47- EU430460; H59- EU430461; H60- EU430464; H65- EU430455, EU430455-2, EU430463, EU430467; H66- EU430465; H73 EU430473; H80- EU430474; H81- EU430475; H86- EU430459; H87- EU430472; H89- EU430462)

iii) Thailand (H34- AF199395, AF199397, AF199402, AF199403, AF199408, AF435602, AF435603; H35- AF435611; H36- AF199398, AF435599, AF435609 H39- AF435608; H40- AF435604; H43- AF435597; H48- AF199396; H49- AF435596, AF435612, AF435624; H50- AF435598; H51- AF199394; H52 AF435614; H61- AF199409; H62- AF199410; H67- AF199393, AF435606, AF435595, Indonesia-DQ907673; H68- AF199400; H69- AF199404, AF435607; H70- AF435605, Vietnam -DQ907674; H76- AF199399, AF435610; H77- AF199401, AF435601; H79- AF435615; H85- AF435613)

iv) Brazil (H44- AF199405; H45- AF199407, AF435630; H53- AF435623, AF435627; H71- AF435625; H72- AF199406)

v) Bangladesh (H33- AF435616, AF435617, AF435620; H54- AF435618; H75- AF435619)

vi) Vanuatu (H55- AF435632, AF435634)

vii) South Korea (H30- AF435637)

vii) Japan (H56- AF435639)

viii) Rio Meta (H46- DQ90767)

ix) Sumatra (H57- DQ907672)

x) Mauritania (H83- DQ907675) (Pacheco *et al.* 2007; Putaporntip *et al.* 2002 & 2000).

Table 4.5. The numbers of isolates present in each of the PvMSP-1_{42} 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)

4.3.4. Phylogenetic tree of *Pvmsp-1*$_{42}$

To investigate PvMSP-1$_{42}$ phylogenetic relationships, we constructed a Neighbor-Joining tree based on the 27 haplotypes described here (Figure 4.4). Interestingly, the haplotypes were clustered into eight groups as shown by the 8 different HVR types (HVR-T1-T8) at PvMSP-1$_{33}$. 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-1*$_{42}$ nucleotide sequences defined 12 distinctive groups (Figure 4.5). As indicated above, the Sri Lankan isolates were clustered in 5 of the 12 groups, with a majority belonging to group 12 while group 6 and 7 included isolates exclusively 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 (Putaporntip *et al.* 2002; Pacheco *et al.* 2007) and a majority of the Sri Lankan isolates from this study, where as the Belem isolate was not clustered in a single group. Group 2, 4 and 11 included isolates from Thailand, while group 5 and 10 were exclusively from India. Group 1 and 8 comprised isolates from Bangladesh and Brazil, respectively. Two isolates from Colombo were clustered in group 9 with 4 Thai, 2 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, of which only 2 sub groups consisted isolates from this study; one with 9 Sri Lankan isolates (02 isolate from Anuradhapura, 05 from Kataragama and 02 from Colombo) closely related to an isolate from Bangladesh, and another with 6 Sri Lankan isolates (2 isolates each belonging to the three test areas) closely related to 4 Indian isolates. The other sub groups contained isolates from Brazil, Thailand and Vanuatu (Figure 4.5).

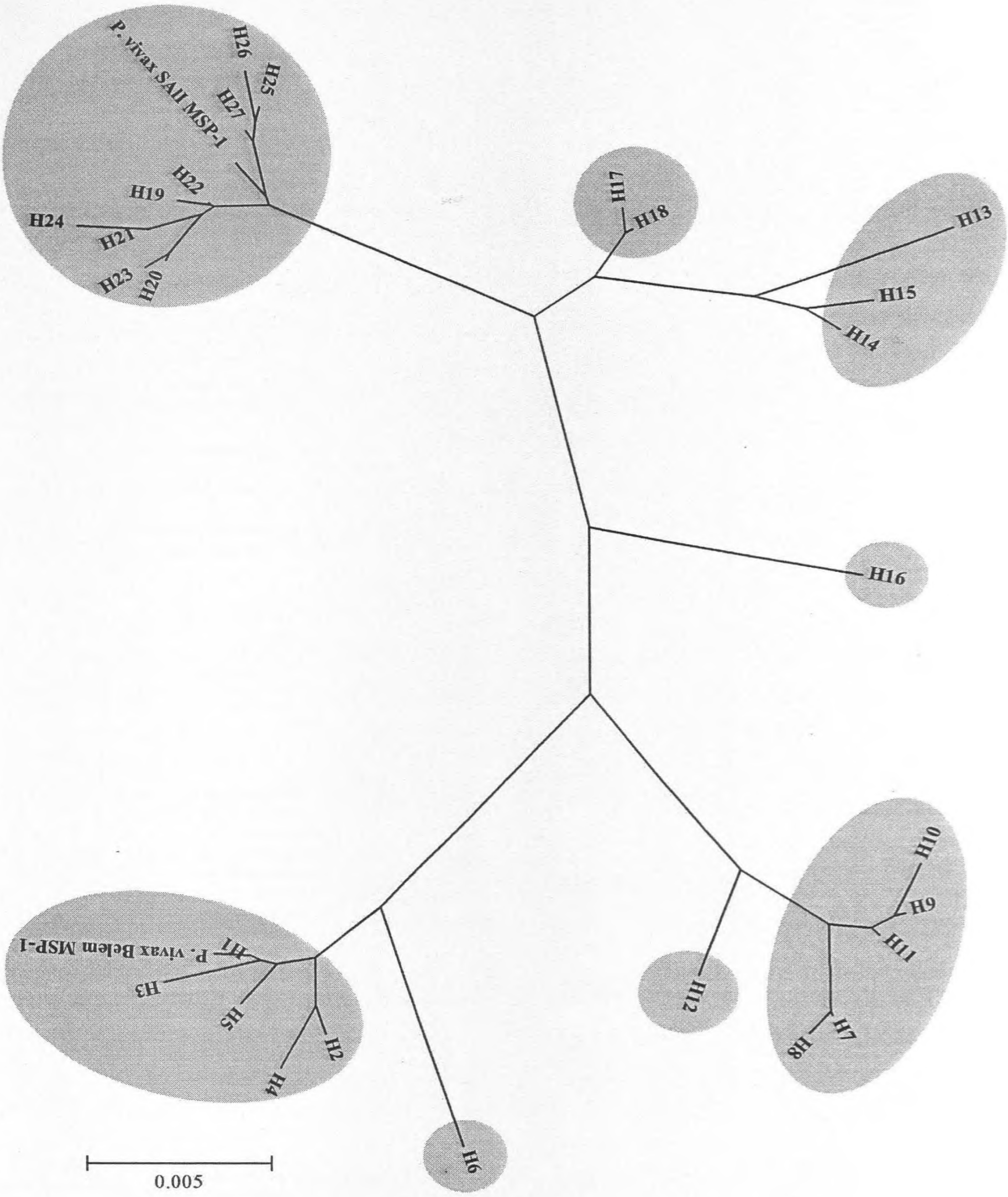


Figure 4.4. Neighbor-Joining tree of PvMSP-1_{42} amino acid sequence of 27 haplotypes observed among 95 Sri Lankan isolates

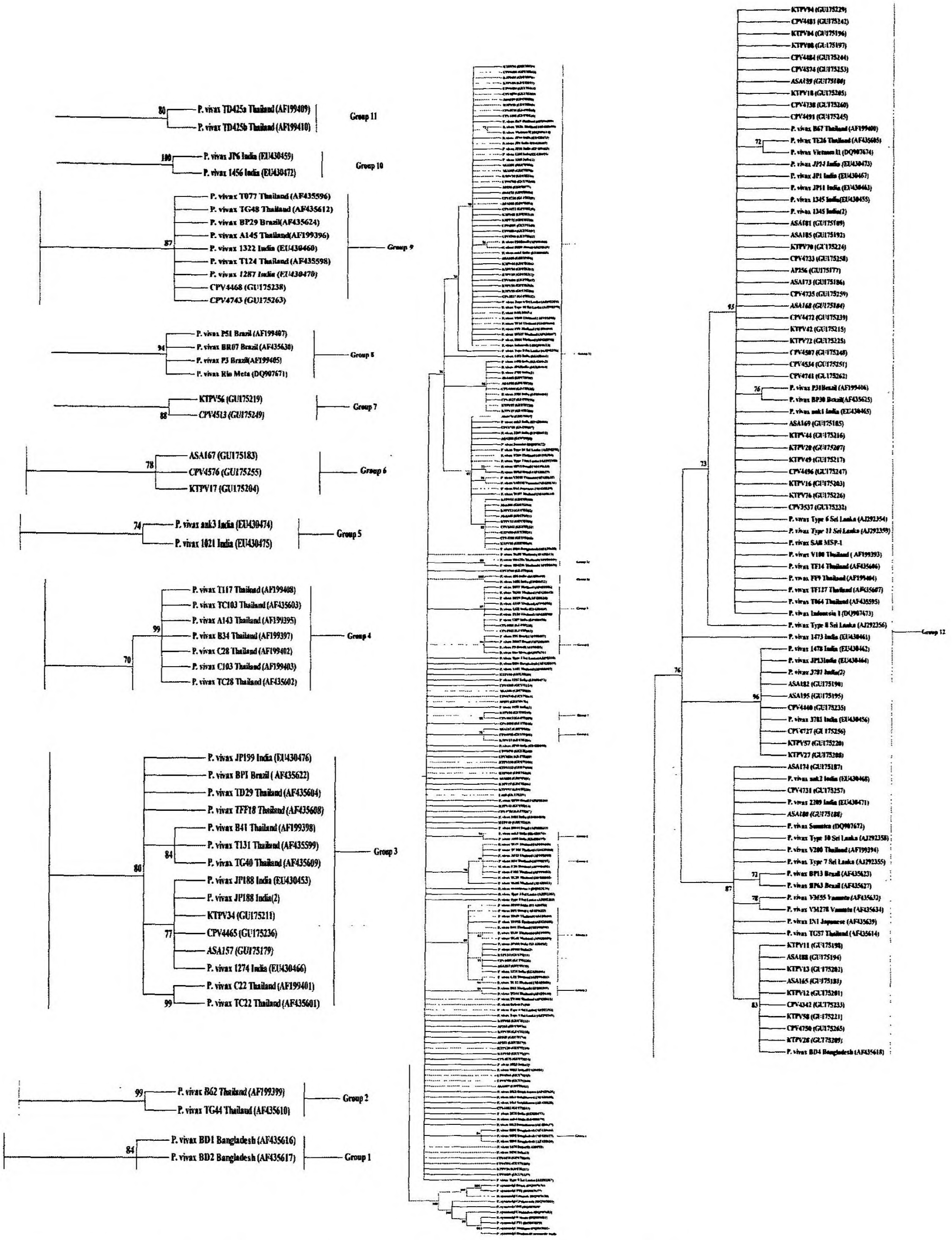


Figure 4.5. Neighbor-joining tree of the *Pvmsp-1*$_{42}$ using Kimura 2 parameter

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

4.3.5. Prediction of B and T-cell epitopes in PvMSP-1_{33} and PvMSP-1_{19}

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, rest of the haplotypes predicted extra B cell epitopes due to amino acid substitutions at several position of the sequence. The 19 kDa fragment predicted three B cell epitopes at amino acid positions 1615, 1657 and 1678.

According to the SYFHEITHI prediction, majority of T cell epitopes were predicted for HLA-DRB1*0101 and DRB1*0401 alleles (14 and 13 number of epitopes, respectively) that would bind to these two alleles with scores between 25- 29 (Table 4.6). Further, four epitopes were predicted at PvMSP-1_{42} for DRB1*0701, while none were predicted for DRB1*1101 allele. Moreover, three eptopes predicted for DRB1*0101 allele corresponding to amino acid positions 1656, 1665 and 1671 belonged to the PvMSP-1_{19} region. Two other epitopes overlapping each other, corresponding to positions 1507 and 1510 of the hypervariable region of PvMSP-1_{33} were common to both HLA-DRB1*0101 and DRB1*0401 alleles, while epitope VNAFNTNITDMLDSR was common to both HLA-DRB1*0101 and DRB1*0701 allele (Table 4.6).

Subsequently, the ProPred MHC class II binding peptide prediction server predicted T cell epitopes on MSP-1_{33} shared by all 27 haplotypes that 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. Moreover, except for a novel epitope manifested on amino acid haplotype H3, common T cell epitopes were predicted for haplotypes H1-H6. Polymorphism in the hypervariable regions of H7-H27 generated diverse predicted T cell epitopes for all of the HLA alleles, except HLA-DRB1*0701. Epitope IGSDSTKTT of the Belem sequence predicted for DRB1*0401 allele was absent in amino acid haplotype H7, due to the polymorphism of G to E. Moreover, two new epitopes were predicted; i) LQKLGSVVS for DRB1*1101 in haplotype H14 and ii) LKKLGNVNS for DRB1*0101 in haplotypes H19- H27 due to polymorphic substitutions (Figure 4.6). While two PvMSP-1_{19} T cell eptiopes were predicted for HLA-DRB1*0101 and for both HLA-DRB1*0401 and 1101, the epitope LLNVQTQLL predicted for HLA-DRB*0701 was positioned at the end of PvMSP-1_{33} and beginning of PvMSP-1_{19} (Figure 4.6).

4.3.6. Homology modelling at PvMSP-1_{42}

Percentage homology of the 27 amino acid haplotypes was analyzed with respect to the amino acid sequence of the PvMSP-1_{42} Belem strain (Figure 4.7). Interestingly, amino acid haplotype H7, which was the predominant haplotype prevalent in Sri Lanka recorded a homology percentage of 92.9% to both the Belem and the Salvador I strains. A homology percentage of 99.7 – 99.1% to the Belem PvMSP-1_{42} strain was observed for amino acid haplotypes H1-H4 which was identical to the Belem strain at the HVR region, while a homology percentage of 91.4 – 92.3% to the Belem strain was observed for amino acid haplotypes H19-H27 which was identical to the Sal I strain at that region. Majority of amino acid haplotypes recorded identical homology percentages to the PvMSP-1_{42} Belem strain with dissimilar amino acid sequences; for e.g. H2 and H4 recorded a homology percentage of 99.1%; H8 and H11 – 92.6%; H9, H13 and H27 – 92.3%, H10, H19, H20, H21 and H26 – 91.7%, H12, H14 and H16 – 93.5%, H15 and H17 – 93.8%, H22 and H25 – 92% (Figure 4.7)

Table 4.5. SYFPEITHI predicted epitopes of the PvMSP-1_{42} region binding to the HLA-DRB1*0101, HLA-DRB1*0401 and HLA-DRB1*0701 molecules

A) SYFPEITHI predicted PvMSP-1_{42} epitope binding to HLA-DRB1*0101

Amino Acid Position	Predicted T cell Epitope	Binding Score
1656	**A** A C **Y** R **Y** L D **G** T E E W R C	29
1671	**L** L T **F** K **E** E G **G** K C V P A S	29
1433	**S** G E **Y** I **I** K D **P** Y K L L D L	28
1507	**D** E E **L** K **K** L G **N** V N S Q D S	28
1510	**L** K K **L** G **N** V N **S** Q D S K K N	28
1628	**L** S Q **L** L **N** V Q **T** Q L L T M S	28
1607	**S** G L **L** E **K** L M **K** S K L I K E	27
1665	**T** E E **W** R **C** L L **T** F K E E G G	27
1454	**I** G S **Y** K **Y** I G **A** S I D M D L	26
1385	**Y** K T **I** K **K** Q L **E** N H V N A F	25
1396	**V** N A **F** N **T** N I **T** D M L D S R	25
1478	**Y** N K **M** G **E** L Y **K** T H L D G V	25
1544	**Q** K E **Y** E **S** L V **S** K V N T Y T	25
1616	**S** K L **I** K **E** N E **S** K E I L S Q	25

B) SYFPEITHI predicted PvMSP-1_{42} epitope binding to HLA-DRB1*0401

1414	**R** N Y **F** L **E** **V** L **N** S D L N P F	28
1533	**L** E K **Y** L **P** **F** L **N** S L Q K E Y	28
1544	**Q** K E **Y** E **S** **L** V **S** K V N T Y T	28
1393	**E** N H **V** N **A** **F** N **T** N I T D M L	26
1415	**N** Y F **L** E **V** **L** N **S** D L N P F K	26
1418	**L** E V **L** N **S** **D** L **N** P F K Y S S	26
1486	**K** T H **L** D **G** **V** K **T** E I K K V E	26
1507	**D** E E **L** K **K** **L** G **N** V N S Q D S	26
1510	**L** K K **L** G **N** **V** N **S** Q D S K K N	26
1534	**E** K Y **L** P **F** **L** N **S** L Q K E Y E	26
1558	**T** D N **L** K **K** **V** I **N** N C Q L E K	26
1623	**K** E I **L** S **Q** **L** L **N** V Q T Q L L	26
1628	**L** S Q **L** L **N** **V** Q **T** Q L L T M S	26

C) SYFPEITHI predicted PvMSP-1_{42} epitope binding to HLA-DRB1*0701

1425	**L** N P **F** K **Y** S S **S** G E Y I I K	30
1427	**P** F K **Y** S **S** S G **E** Y I I K D P	30
1457	**Y** K Y **I** G **A** S I **D** M D L A T A	28
1396	**V** N A **F** N **T** N I **T** D M L D S R	26

DRB1_0101:MYKTIKKQLENHVNAFNTNITDMLDSRLKKRNYFLEVLNSDLNPFKYSPSGEYIIKDPYKLLDLEKKKKLLGSYKYIGASIDKDLATANDGVTYYNKM
GELYKTHLTAVNEEVKKVEADIKAEDDKIKKIGSDSTKTTEKTQSMAKKAELEKYLPFLNSLQKEYESLVSKVNTYTDNLKKVINNCQLEKKEAEITVKKLQDYNK
MDEKLEEYKKSEKKNEVKSSGLLEKLMKSKLIKENESKEILSQLLNVQTQLLTMSSEHTCIDTNVPDNAACYRYLDGTEEWRCLLTFKEEGGKCVPASNVTCKDNN
GGCAPEAECKMTDSNKIVCKCTKEGSEPL

DRB1_0401:MYKTIKKQLENHVNAFNTNITDMLDSRLKKRNYFLEVLNSDLNPFKYSPSGEYIIKDPYKLLDLEKKKKLLGSYKYIGASIDKDLATANDGVTYYNKM
GELYKTHLTAVNEEVKKVEADIKAEDDKIKKIGSDSTKTTEKTQSMAKKAELEKYLPFLNSLQKEYESLVSKVNTYTDNLKKVINNCQLEKKEAEITVKKLQDYNK
MDEKLEEYKKSEKKNEVKSSGLLEKLMKSKLIKENESKEILSQLLNVQTQLLTMSSEHTCIDTNVPDNAACYRYLDGTEEWRCLLTFKEEGGKCVPASNVTCKDNN
GGCAPEAECKMTDSNKIVCKCTKEGSEPL

DRB1_0701:MYKTIKKQLENHVNAFNTNITDMLDSRLKKRNYFLEVLNSDLNPFKYSPSGEYIIKDPYKLLDLEKKKKLLGSYKYIGASIDKDLATANDGVTYYNKM
GELYKTHLTAVNEEVKKVEADIKAEDDKIKKIGSDSTKTTEKTQSMAKKAELEKYLPFLNSLQKEYESLVSKVNTYTDNLKKVINNCQLEKKEAEITVKKLQDYNK
MDEKLEEYKKSEKKNEVKSSGLLEKLMKSKLIKENESKEILSQLLNVQTQLLTMSSEHTCIDTNVPDNAACYRYLDGTEEWRCLLTFKEEGGKCVPASNVTCKDNN
GGCAPEAECKMTDSNKIVCKCTKEGSEPL

DRB1_1101:MYKTIKKQLENHVNAFNTNITDMLDSRLKKRNYFLEVLNSDLNPFKYSPSGEYIIKDPYKLLDLEKKKKLLGSYKYIGASIDKDLATANDGVTYYNKM
GELYKTHLTAVNEEVKKVEADIKAEDDKIKKIGSDSTKTTEKTQSMAKKAELEKYLPFLNSLQKEYESLVSKVNTYTDNLKKVINNCQLEKKEAEITVKKLQDYNK
MDEKLEEYKKSEKKNEVKSSGLLEKLMKSKLIKENESKEILSQLLNVQTQLLTMSSEHTCIDTNVPDNAACYRYLDGTEEWRCLLTFKEEGGKCVPASNVTCKDNN
GGCAPEAECKMTDSNKIVCKCTKEGSEPL

Figure 4.6. Predicted T cell epitopes of the PvMSP-1_{42} Belem strain obtained for the HLA-DRB1*0101, HLA-DRB1*0401, HLA-DRB1*0701 and HLA-DRB1*1101 alleles

The hypervariable region of PvMSP-1_{42} is underlined and the polymorphic epitopes are circled.

	Belem	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20	H21	H22	H23	H24	H25	H26	H27
Belem	ID	0.997	0.991	0.994	0.991	0.985	0.967	0.929	0.926	0.923	0.917	0.926	0.935	0.923	0.935	0.938	0.935	0.938	0.932	0.917	0.917	0.917	0.920	0.914	0.914	0.920	0.917	0.923
H1	0.997	ID	0.994	0.997	0.994	0.988	0.970	0.932	0.929	0.926	0.920	0.929	0.938	0.926	0.938	0.941	0.938	0.941	0.935	0.920	0.920	0.920	0.923	0.917	0.917	0.923	0.920	0.926
H2	0.991	0.994	ID	0.991	0.988	0.994	0.976	0.938	0.935	0.932	0.926	0.935	0.943	0.932	0.932	0.935	0.932	0.935	0.929	0.920	0.920	0.920	0.923	0.917	0.917	0.917	0.914	0.920
H3	0.994	0.997	0.991	ID	0.991	0.985	0.967	0.929	0.926	0.923	0.917	0.926	0.935	0.923	0.935	0.938	0.941	0.938	0.932	0.923	0.917	0.917	0.920	0.914	0.914	0.926	0.923	0.923
H4	0.991	0.994	0.988	0.991	ID	0.988	0.970	0.926	0.923	0.926	0.920	0.929	0.938	0.929	0.932	0.938	0.932	0.935	0.932	0.920	0.920	0.920	0.923	0.917	0.917	0.917	0.914	0.920
H5	0.985	0.988	0.994	0.985	0.988	ID	0.976	0.932	0.929	0.938	0.932	0.935	0.943	0.938	0.926	0.929	0.926	0.929	0.923	0.920	0.926	0.920	0.923	0.923	0.917	0.911	0.908	0.914
H6	0.967	0.970	0.976	0.967	0.970	0.976	ID	0.949	0.946	0.949	0.943	0.952	0.938	0.932	0.943	0.929	0.926	0.929	0.920	0.923	0.923	0.923	0.926	0.920	0.920	0.914	0.911	0.917
H7	0.929	0.932	0.938	0.929	0.926	0.932	0.949	ID	0.997	0.994	0.988	0.997	0.982	0.926	0.970	0.929	0.932	0.935	0.920	0.932	0.932	0.932	0.935	0.929	0.929	0.929	0.926	0.932
H8	0.926	0.929	0.935	0.926	0.923	0.929	0.946	0.997	ID	0.991	0.991	0.994	0.979	0.923	0.967	0.926	0.929	0.932	0.923	0.929	0.929	0.929	0.932	0.926	0.932	0.926	0.923	0.929
H9	0.923	0.926	0.932	0.923	0.926	0.938	0.949	0.994	0.991	ID	0.994	0.997	0.982	0.932	0.964	0.923	0.926	0.929	0.914	0.932	0.938	0.932	0.935	0.935	0.929	0.923	0.920	0.926
H10	0.917	0.920	0.926	0.917	0.920	0.932	0.943	0.988	0.991	0.994	ID	0.991	0.976	0.926	0.958	0.917	0.920	0.923	0.920	0.926	0.932	0.932	0.929	0.935	0.935	0.917	0.920	0.920
H11	0.926	0.929	0.935	0.926	0.929	0.935	0.952	0.997	0.994	0.997	0.991	ID	0.985	0.929	0.967	0.926	0.929	0.932	0.917	0.935	0.935	0.935	0.938	0.932	0.932	0.926	0.923	0.929
H12	0.935	0.938	0.943	0.935	0.938	0.943	0.938	0.982	0.979	0.982	0.976	0.985	ID	0.929	0.952	0.920	0.923	0.926	0.914	0.932	0.932	0.932	0.935	0.929	0.929	0.923	0.920	0.926
H13	0.923	0.926	0.932	0.923	0.929	0.938	0.932	0.926	0.923	0.932	0.926	0.929	0.929	ID	0.938	0.985	0.970	0.973	0.976	0.949	0.955	0.949	0.952	0.952	0.946	0.941	0.938	0.943
H14	0.935	0.938	0.932	0.935	0.932	0.926	0.943	0.970	0.967	0.964	0.958	0.967	0.952	0.938	ID	0.952	0.961	0.964	0.943	0.955	0.955	0.955	0.958	0.952	0.952	0.958	0.955	0.961
H15	0.938	0.941	0.935	0.938	0.938	0.929	0.929	0.929	0.926	0.923	0.917	0.926	0.920	0.985	0.952	ID	0.985	0.988	0.991	0.952	0.952	0.952	0.955	0.949	0.949	0.955	0.952	0.958
H16	0.935	0.938	0.932	0.941	0.932	0.926	0.926	0.932	0.929	0.926	0.920	0.929	0.923	0.970	0.961	0.985	ID	0.997	0.976	0.967	0.961	0.961	0.964	0.958	0.958	0.970	0.967	0.967
H17	0.938	0.941	0.935	0.938	0.935	0.929	0.929	0.935	0.932	0.929	0.923	0.932	0.926	0.973	0.964	0.988	0.997	ID	0.979	0.964	0.964	0.964	0.967	0.961	0.961	0.967	0.964	0.970
H18	0.932	0.935	0.929	0.932	0.932	0.923	0.920	0.920	0.923	0.914	0.920	0.917	0.914	0.976	0.943	0.991	0.976	0.979	ID	0.949	0.949	0.955	0.952	0.952	0.958	0.952	0.955	0.955
H19	0.917	0.920	0.920	0.923	0.920	0.920	0.923	0.932	0.929	0.932	0.926	0.935	0.932	0.949	0.955	0.952	0.967	0.964	0.949	ID	0.994	0.994	0.997	0.991	0.991	0.991	0.988	0.988
H20	0.917	0.920	0.920	0.917	0.920	0.926	0.923	0.932	0.929	0.938	0.932	0.935	0.932	0.955	0.955	0.952	0.961	0.964	0.949	0.994	ID	0.994	0.997	0.997	0.991	0.985	0.982	0.988
H21	0.917	0.920	0.920	0.917	0.920	0.920	0.923	0.932	0.929	0.932	0.932	0.935	0.932	0.949	0.955	0.952	0.961	0.964	0.955	0.994	0.994	ID	0.997	0.997	0.997	0.985	0.988	0.988
H22	0.920	0.923	0.923	0.920	0.923	0.923	0.926	0.935	0.932	0.935	0.929	0.938	0.935	0.952	0.958	0.955	0.964	0.967	0.952	0.997	0.997	0.997	ID	0.994	0.994	0.988	0.985	0.991
H23	0.914	0.917	0.917	0.914	0.917	0.923	0.920	0.929	0.926	0.935	0.935	0.932	0.929	0.952	0.952	0.949	0.958	0.961	0.952	0.991	0.997	0.997	0.994	ID	0.994	0.982	0.985	0.985
H24	0.914	0.917	0.917	0.914	0.917	0.917	0.920	0.929	0.932	0.929	0.935	0.932	0.929	0.946	0.952	0.949	0.958	0.961	0.958	0.991	0.991	0.997	0.994	0.994	ID	0.982	0.985	0.985
H25	0.920	0.923	0.917	0.926	0.917	0.911	0.914	0.929	0.926	0.923	0.917	0.926	0.923	0.941	0.958	0.955	0.970	0.967	0.952	0.991	0.985	0.985	0.988	0.982	0.982	ID	0.997	0.997
H26	0.917	0.920	0.914	0.923	0.914	0.908	0.911	0.926	0.923	0.920	0.920	0.923	0.920	0.938	0.955	0.952	0.967	0.964	0.955	0.988	0.982	0.988	0.985	0.985	0.985	0.997	ID	0.994
H27	0.923	0.926	0.920	0.923	0.920	0.914	0.917	0.932	0.929	0.926	0.920	0.929	0.926	0.943	0.961	0.958	0.967	0.970	0.955	0.988	0.988	0.988	0.991	0.985	0.985	0.997	0.994	ID

Figure 4.7. Identity matrix generated for the 27 amino acid haplotypes of PvMSP-1_{42} among Sri Lankan *P. vivax* infected isolates

The amino acid sequences were compared with the Belem sequence of PvMSP-1_{42} (Accession No. AF435594; Putaporntip *et al.* 2002).

4.4. Discussion

The merozoite surface protein-1_{42} (MSP-1_{42}) 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, genetic differentiation between different populations and natural selection (Cole-Tobian and King, 2003). This study was undertaken to investigate the existing polymorphism in the *Pvmsp-1_{42}* gene in *P. vivax* clinical isolates of Sri Lanka and to elaborate the mechanisms generated to maintain polymorphism and the alteration of immunological recognition due to polymorphism.

Evidence for positive selection can be found by increased levels of nucleotide diversity between alleles or haplotypes in a species. Among the three study areas of Sri Lanka, the nucleotide diversity at *Pvmsp-1_{33}* was low in Anuradhapura followed by Colombo compared to Kataragama. During the collection of patient blood samples the transmission of *P. vivax* malaria within the three study areas of Sri Lanka differed, where the annual parasite incidence per 1000 due to *P. vivax* was 0 – 1.25 in Colombo and 10-20 in Kataragama and Anuradhapura (Briët *et al.* 2003). Therefore, transmission of the parasite may be a governing factor for the foreseen genetic diversity at *Pvmsp-1_{33}*. However, though *Pvmsp-1_{19}* recorded elevated nucleotide diversity in Colombo compared to the two endemic areas, at the amino acid level these isolates in all three study areas were identical, subsequently highlighting this region as potential component for a subunit vaccine construct.

The level of polymorphism observed in the *Pvmsp-1_{42}* gene in Sri Lanka was low compared to *msp-3α* (π= 0.059) (Wickramarachchi *et al.* 2010), but higher than other merozoite proteins *dbpII* (π= 0.0098) and *ama-1* (π=0.0095) (P. V. Udagama-Randeniya, unpublished data). In Sri Lanka *Pvmsp-1_{33}* exhibited greater genetic diversity due to 62 point mutations and the 38 highly polymorphic amino acid region defined as the hypervariable region (HVR), confirming previous reports (Pacheco *et al.* 2007; Thakur *et al.* 2008a). Subsequently, Tanabe *et al.* (2007) demonstrated that the highest Dn/Ds ratio (5.6) was observed in the N-terminal 33 kD fragment which represents the secondary processing product of the C-terminal 42 kD region of MSP-1, which was confirmed by the current study using the sliding window analysis.

Porto *et al.* (1992) demonstrated that sequence analysis of a polymorphic region between ICB5 and ICB6 of the *msp-1* gene revealed the existence of three types of polymorphisms.

Two were almost identical either to the Belem or the Sal I strain. The third polymorphic type was a recombination of the two. The same three types were also observed at PvMSP-1_{42} by Putaporntip and co-workers, (2000). Though, none of the 27 PvMSP-1_{42} amino acid haplotypes were identical either to 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 as well as by the homology matrix analysed with reference to the Belem strain. However, a previous study by Manamperi, (2002), revealed classical Belem and Sal I strains of Merozoite Surface Protein-1_{42} from 19 *P. vivax* isolates collected from Kataragama, Sri Lanka.

Four of the nine hypervariable region (HVR) types from Sri Lankan isolates recorded by Manamperi, (2002) were observed in the current study. Nine of the 10 HVR types observed in local isolates were also found in global isolates, and is 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 consisted of Asian origin, with the exception of 2 Brazilian isolates of the T2 type, and also that the 2 new HVR types (T11 and T12) were clustered into two clear groups. It was shown that the *P. vivax* population of Asia was ancestral to Brazilian populations and that recombination in these unique sequences predated subdivision of *P. vivax* populations in the new world (Putaporntip *et al.* 2002). This may explain the 2 Brazilian isolates mingling with those of the Asian origin of the HVR-T2 type.

Further the recombination sites distributed across *Pvmsp-1_{42}* were not uniform corroborating previous findings (Putaporntip *et al.* 2002), with the highest number of recombination events (Rm) at *Pvmsp-1_{33}*. This explains the HVR types T3-T7 and the 19 haplotypes (H71-84) that were generated solely due to intragenic recombination and further confirms that the extensive polymorphism present at PvMSP-1_{33} 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 further clarify that new PvMSP-1_{42} alleles could be generated by recombination events even in areas with low transmission of malaria. Further, long-lasting and recurrent parasitemia is often associated

with relapse, a characteristic of *P. vivax* infection, that may contribute to an increased rate of heterozygote formation and subsequent interallele recombination in the *Anopheline* mosquito vector (Putaporntip *et al.* 2002).

The significance of certain haplotypes common to Colombo and to those from the two endemic areas is possible due to the following factors; i) patients residing in Colombo contracted the disease during visits to endemic areas of the country with *P. vivax* transmission due to occupational commitments (Fonseka and Mendis, 1987; Briet *et al.* 2003), where the origin of these infections was traced to eleven administrative districts (Ampara, Anuradhapura, Hambantota, Jaffna, Kurunagala, Monaragala, Mullaitivu, Pollonnaruwa, Puttalum, Ratnapura and Vavuniya) 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. According to Holsinger and Weir, (2009), F_{ST} is directly related to the variance in allele frequency among populations and, conversely, to the degree of resemblance among individuals within populations. If F_{ST} is small, it means that the allele frequencies within each population are similar; if it is large, it means that the allele frequencies are different. If natural selection favours one allele over others at a particular locus in some populations, the F_{ST} at that locus will be larger than at loci in which among-population differences are purely a result of genetic drift. The phylogenetic tree further confirmed the low genetic differentiation due to migration between Sri Lanka and neighbouring India with the majority of common amino acid haplotypes between the two, as well as the marked genetic differentiation between Sri Lanka and Bangladesh shown by the separate clustering of 3 of the 5 Bangladesh isolates. On the contrary, one of the two remaining isolates clustered uniquely with 9 Sri Lankan isolates from this study, while the other was closely related to a Thai and a Sri Lankan isolate (Manamperi, 2002). Overall, the phylogenetic tree showed no evidence of geographic clustering of isolates, rather the interchange of *P. vivax* strains due to migration of individuals and malaria transmitting vectors was evident.

In Sri Lankan isolates, PvMSP-1_{33} was subjected to positive selection, where a high rate of non-synonymous substitutions relative to synonymous ones occurred, presumably to avoid host immune pressure. Consequently, these polymorphisms would be maintained in *P. vivax* populations, and host immune responses may play a role in maintaining polymorphism of *P. vivax* MSP-1 alleles. However, for *P. falciparum* MSP-1_{33} was under purifying selection (Pacheco *et al.* 2007). This suggests the different roles of this antigen in the host–parasite

immune interaction in each of the major human malarial parasites. Two mechanisms that enable PvMSP-1_{33} to maintain positive selection in the local isolates are (i) presence of three of the five positive selection sites essential for immune evasion by the parasite (Tanabe *et al.* 2007), at amino acid positions 1490 (D/T), 1491 (G/A) and 1494 (T/A/E) in the 33 kDa fragment, and (ii) clusters of non-synonymous substitutions occurring on the predicted T cell epitopes in all 27 amino acid haplotypes at PvMSP-1_{33} that would induce differential binding of these polymorphic peptides to class II MHC alleles. Due to amino acid polymorphism occurring in these epitopes, immune responses may differ or completely arrest assisting the parasite to escape the host immune system (Tanabe *et al.* 2007). The uses of bioinformatics tools for the prediction of epitopes are useful, but they are to be used with caution, corroborating their results experimentally. A recent study by Patarroyo and co-workers (2008) demonstrated that some data obtained from experimental studies for HLA alleles 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. Moreover, some T cell epitopes predicted by the SYFPEITHI software were different to those predicted by the ProPred MHC class II binding peptide prediction server.

Polymorphism located between regions of peptides with high specific binding activity (HSBA) to reticulocytes had previously been shown (Rodríguez *et al.* 2002; Espinosa *et al.* 2003). Indeed, there is a 105 bp fragment with high polymorphism located between regions where peptides with high specific binding activity (HSBA) to reticulocytes have been found. Some of these high activity binding peptides (HABPs) are located close to the hypothesized 42 and 19 kDa fragment cleavage sites MSP-1, suggesting that these sequences have an important role in target cell attachment and in the invasion process of PvMSP-1. This overall pattern indicated that these HSBA regions are under selective constraints to accumulate amino acid replacements and as a result, these could be a valuable target for a vaccine against *P. vivax* (Espinosa *et al.* 2003).

Extensive genetic diversity at the *Pvmsp-1_{33}* locus in the island where low transmission and unstable malaria conditions persist highlight both interallelic and intragenic recombination as critical factors for the origin of new PvMSP-1_{42} amino acid haplotypes in the local *P. vivax* populations. Moreover, positive selection for polymorphism acting on PvMSP-1_{33} 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-1_{42} in Sri Lanka (Wickramarachchi *et al.* 2007).

A single amino acid variant occurring at position 1709 (K/E) in the 19 kDa fragment was observed from 04 Thai and a single Sri Lankan isolate in previous studies (Manamperi, 2002; Putaporntip *et al.* 2002 & 2000). Conversely, with the exception of a single recombination event, PvMSP-1_{19} at the amino acid level was highly conserved in all 95 Sri Lankan isolates tested and the rest of the global isolates analyzed, with confirmed lack of T cell epitope variations. Since the PvMSP-1_{19} domain is cystein rich, with multiple disulphide bonds forming two EGF like domains, B cell epitopes appear to be almost exclusively conformational opposed to T cell epitopes which may be poorly processed (Soares *et al.* 1997). Furthermore, as reported previously by different groups, the 19 kDa C-terminal region of MSP-1 is a highly immunogenic conserved region of *P. vivax* (Ak *et al.* 1998; Soares *et al.* 1997; Park *et al.* 2001; Morais *et al.* 2005; Wickramarachchi *et al.* 2007). Indeed, a baculovirus recombinant version of *P. cynomolgi* MSP-1_{19}, which is closely related to *P. vivax*, was highly protective in primate vaccination trials carried out in Sri Lanka (Perera *et al.* 1998). Moreover, that PvMSP-1_{19} is under purifying selection, and the protective nature of the natural antibody response against the 19 kDa fragment previously demonstrated the marked switch of anti-PvMSP-1_{19} 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-1_{19} to be effective against *P. vivax* infections in Sri Lanka. Thus, it is prudent to suggest at this point that future experimental procedures be undertaken to study predicted T and B cell epitopes found on the PvMSP-1_{19} region, as the presence of such universal epitopes will be enormously important for a potential vaccine candidate.

CHAPTER 5

Strain-specific immunity to *Plasmodium vivax* Apical Membrane Antigen-1 (PvAMA-1) and Merozoite Surface Protein-1 (PvMSP-1) in Sri Lanka

5.1. Introduction

5.1.1. Immuno-epidemiology of AMA-1 and MSP-1

Importance of immuno-epidemiological studies at the individual level can reveal how factors such as frequency of infection or genetic variation affect levels of immunity and, at the community level, can reveal how immune responses influence the prevalence of infection and the burden of disease (Kwiatkowski, 2005).

Immuno-epidemiological studies have shown a high prevalence of antibodies against the C-terminal of PvMSP-1 and an increase of IgG prevalence with exposure to *P. vivax* malaria in endemic regions of South America and Turkey (Soares *et al.* 1999; Zeyrek *et al.* 2008). Serological studies have provided significant evidence to suggest that immune responses directed against the C-terminus of MSP-1 (MSP-1_{42} and MSP-1_{19}) are associated with immunity in preclinical models (Long *et al.* 1994; Egan *et al.* 1999; Darko *et al.* 2005; Parkkinen *et al.* 2006), and in individuals residing in endemic areas (Egan *et al.* 1999; John *et al.* 2004). Wickramarachchi and co-workers (2007) demonstrated a concurrent reduction of IgM with functionally important IgG1 and IgG3 isotypes dominating the anti-MSP-1_{19} antibody response with increasing exposure to malaria in two endemic areas, Anuradhapura and Kataragama, of Sri Lanka, while, in contrast MSP-1_{42} antibodies principally reflected a primary IgM response irrespective of the patient's previous exposure. Therefore, the immature antibody response directed against MSP-1_{42} polymorphic region may interfere with the MSP-1_{19} "protective" isotype shift, and suggested that immunological "cross talk" between epitopes of PvMSP-1_{42} and $_{19}$ may interfere with the development of a mature IgG dominated protective antibody response, which may complicate vaccine development. Further, recent studies have documented that patients living in endemic areas (Thailand and Turkey) tend to develop anti-PvMSP-1_{19} IgG antibody responses over many episodes and with age (Pitabut *et al.,* 2007; Zeyrek *et al.,* 2008).

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, 1996).

5.1.2. Immune responses against specific domains of AMA-1 and MSP-1

The use of individual domains or sub-domains of the ectoplasmic region of AMA-1 could lead to simpler and possibly more stable AMA-1 based vaccine products (Chesne-Seck *et al.* 2005). For example, antibodies that were affinity purified from the plasma of a donor representing a malaria endemic region showed significant inhibition of erythrocyte invasion to two different strains of the recombinant PfAMA-1 domain III, demonstrating the presence of protective epitopes in this domain (Nair *et al.* 2002). However, a recent study in rabbits using different recombinant PfAMA-1 domain combinations as immunogens suggested otherwise, where individual domains were not effective in inducing invasion inhibitory antibodies rather, only the double domain I/II combination induced growth-inhibitory antibody levels comparable to the complete ectoplasmic region (Lalitha *et al.* 2004). Interestingly, Múfalo and co-workers (2008) expressed each domain separately or in combination (DI-II or DII-III) as bacterial recombinant proteins to map immuno dominant epitopes within the PvAMA-1 ectodomain, using sera of *P. vivax* infected individuals collected from endemic regions of Brazil, or antibodies raised in immunized mice. The frequencies of responders against recombinant protein representing DII were higher than to the other domains and similar to the ones observed against the PvAMA-1 ectodomain. Moreover, ELISA inhibition assays using the PvAMA-1 ectodomain as substrate revealed the

presence of many common epitopes within DI-II recognized by human immune antibodies, elucidating that DII is particularly immunogenic during natural human infections, thus implicating the inclusion of this region in an experimental sub-unit vaccine to prevent vivax malaria. Moreover, the activity of the invasion-inhibitory monoclonal antibody 4G2, which is recognised by a reduction-sensitive epitope located at the base of the PfAMA-1 domain II loop, requires the presence of domain I (Pizarro *et al.* 2005). However, since the domain II loop contains a human T-cell epitope as well (Lal *et al.* 1996) and carries no known polymorphisms, peptide mimics of this region would be of particular interest for vaccine development.

The sequentially processed 42 kDa and 19 kDa fragments of the merozoite surface protein-1 (MSP-1) provided evidence *in vitro* and *in vivo* studies that, these two fragments are the targets of protective immunity against asexual blood stages of malaria parasites in both *P. falciparum* and *P. vivax*. Studies on natural antibody responses against the C-terminal proteolytically processed fragments were recorded in different endemic areas of the world; antibody responses to C-terminal PfMSP-1 were associated with a reduced risk of clinical malaria in hyperendemic areas such as Gambia, PNG and Senegal (Shai *et al.* 1995; Al-Yaman *et al.* 1996; Perraut *et al.* 2005), while a high prevalence of antibodies against C-terminal regions of PvMSP-1 and an increase of IgG prevalence with exposure to *P. vivax* malaria was also demonstrated in individuals living in endemic regions of South America and Sri Lanka where low malaria transmission persists (Soares *et al.* 1999; Manamperi, 2002; Wickramarachchi *et al.* 2007). A study by Nogueira and colleagues (2006) was the first to demonstrate an association of clinical protection and reduced risk of infection with naturally acquired IgG antibodies, predominantly of the IgG3 subclass, against PvMSP-1. However, this association was with the N terminus but not the C terminus of PvMSP-1, in spite of the latter being the most immunogenic portion of the molecule and the only portion capable of boosting natural infections.

5.1.3. Polymorphism and strain-specific immunity acting on AMA-1 and MSP-1

A main obstacle for the acquisition of anti-malarial immunity is the polymorphism in potential target antigens, which enable parasites to evade immune responses elicited by past exposure to variant forms of the same antigen (Ferreira *et al.* 2004). Polymorphisms likely arise as a consequence of immune-mediated selection since host responses can be more

effective against parasites of the immunizing strain than against different strains (strain-specific immunity) (Mendis *et al.* 1991; Anders *et al.* 1998; Cheesman *et al.* 2009).

As a consequence of natural selection, antigens located on the surface or in the apical organelles of the *Plasmodium* merozoite are highly polymorphic, indicative of immune responses against these antigens to be effective in controlling parasite growth (Conway and Polley, 2002). However, antigenic diversity can further be a major obstacle for the development of effective vaccines based on these antigens. 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). Several proteins of malaria parasites elucidating such properties have been identified, of which prominent are MSP-1 and AMA-1 proteins that are products of highly polymorphic genes of the malaria parasite, which is evident to trigger "strain-specific" protective immunity against blood stage malarial infection (Crewther *et al.* 1996; Renia *et al.* 1997; Rotman *et al.* 1999; Genton *et al.* 2002).

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.

Comparison of the naturally acquired antibody responses against three polymorphic domains and the highly conserved C-terminal domain of PvMSP-1 in a cohort of malaria-exposed rural Amazonians were analysed by measuring specific antibodies in the absence of *P. vivax* infection and during subsequent infections. Sequences of the variable domains of PvMSP-1 used in serology were characterized in local parasites circulating before and during the follow-up to confirm that the variants expressed as recombinant antigens matched those to which the subjects were exposed (Bastos *et al.* 2007). Except for the invariant C-terminal domain PvMSP-1_{19}, the rest of the variant recombinants were poorly recognized. Although the proportion of responders to PvMSP-1 variants increased substantially during subsequent acute *P. vivax* infections, the specificity of IgG antibodies did not necessarily match the PvMSP-1 variant(s) found in parasites of the current infection.

A cross sectional study conducted by Manamperi (2002) with a limited number of *P. vivax* infected acute patients (N=19) from a malaria endemic region of Sri Lanka revealed that anti-sera reactivity to multiple epitopes expressed in the PvMSP-1_{33} hypervariable region of some individuals cross reacted with peptides representing both the Belem (del Portillo *et al.* 1991) and the Salvador I (Gibson *et al.* 1992) strains of *P. vivax*. It was evident that antibody reactivity against this region correlated with the homology of the allele in the current infection.

5.1.4. Strain-specific versus strain-transcending (cross reactive) immune responses to malarial antigens

Both strain-specific and strain-transcending (cross reactive) immunity was documented in rodent and simian models and in humans, in the laboratory as well as in the field (Doolan *et al.* 2009). Significant strain-specific and cross reactive inhibition of parasite multiplication was observed when homologous and heterologous sera from Gambian children were assessed for any inhibitory effects on parasite growth (Reviewed in Doolan *et al.* 2009). Despite *in vitro* and *in vivo* support for the strain-specificity of immune responses, there was evidence that naturally exposed individuals develop cross reactive antibodies which recognise an increasingly broad array of *P. falciparum* isolates with increasing age or exposure (Doolan *et al.* 2009). Chattopadhyay *et al.* (2003) demonstrated the presence of cross reactive antibodies against variant surface antigens among adults in a hyperendemic area in India. These data

were consistent with the acquisition of immunity against antigenically diverse strains with exposure.

Point mutations occur in all regions of the gene and numbers of amino acid repeats occur in these polymorphic regions (Franks *et al.* 2003). Therefore, analysis of responses to polymorphic sequences is difficult, as the infecting parasite genotype of a patient is rarely known, and so the antibodies generated against that particular parasite genotype may also be obscure. Nevertheless, sera from a high proportion of both children and adults did recognise recombinant proteins representing individual polymorphic sequence strains, indicating either that these strains were common in the parasite population or that they contained epitopes that were cross reactive with other sequences (Taylor *et al.* 1995).

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. Manamperi (2002) recorded that, the immune response against the PvMSP-1_{42} region correlated with the current infection allele, regardless of previous infection. However, this response was characterised by multiple cross reactive B cell epitopes in the hypervariable block of MSP-1_{42} suggestive of an immature response.

5.1.5. Mixed allele malaria vaccines

Sequence polymorphisms were directly implicated in antigenic escape (Kennedy *et al.* 2002; Healer *et al.* 2004; Saul, 2007b). In malaria endemic areas, immunity is acquired gradually, over a period of time due to repeated exposure, which is a requirement to generate an effective response against a repertoire of parasite strains (Day and Marsh, 1991; reviewed in Barclay *et al.* 2008). The existence of antigenic polymorphism is therefore of considerable concern to malaria vaccine development, as it implies that, single antigen vaccines will face problems inducing protective immunity against polymorphic targets (Hodder *et al.* 2001; Fluck *et al.* 2004; Genton *et al.* 2003; Genton and Reed, 2007). One approach to minimize vaccine induced strain specificity is to design vaccines with a combination of more than one allele of an antigen (Hoffmann *et al.* 1996; Bolad and Berzins, 2000; Richie & Saul, 2004;

Polley *et al.* 2007). However, inclusions of more than one allelic form of an antigen may not be sufficient to overcome substantial polymorphisms (Saul, 2007b), and there is little experimental evidence to show that multi-allelic vaccines actually afford the host more protection from morbidity than single antigen vaccines. Furthermore, selection of antigen variants is not the only evolution that could occur in response to widespread vaccination.

Theoretically, vaccination has the potential to cause evolutionary change in parasite virulence (parasite-induced host damage) by altering natural selection that acts on parasite populations (Gandon & Day, 2007). In experimental evolution experiments, the rodent malaria *Plasmodium chabaudi*, became more rapidly virulent when serially passaged through mice previously immunized with live parasites, as more aggressive variants are less well controlled by immunity (Mackinnon & Read, 2004). Understanding how vaccines will alter gene frequencies in malaria parasite populations to date is vague. Evidence for selection in the field was documented from a small phase 1-2b trial of the "Combination B" blood stage malaria vaccine (Genton *et al.* 2002). This vaccine contained a single antigen from each of the three polymorphic loci of *P. falciparum*. One of these loci, Merozoite Surface Protein-2 (MSP-2), is dimorphic, with each parasite having an allele from one of the two allelic families (labelled 3D7 and FC27). The MSP-2 allele in the Combination B vaccine was from the 3D7 family. Among parasites subsequently acquired by vaccines, 3D7-type alleles were rarer than in individuals given a placebo. Vaccination group was thus selected against the variant contained in the vaccine. Interestingly, the FC27 allelic family is associated with more virulent infections (Engelbrecht *et al.* 1995). Therefore, it is not clear whether the vaccine-imposed selection was due to immune specificity or whether the vaccine was less good at controlling more virulent infections (Fluck *et al.* 2004 & 2007). Allelic replacement experiments have directly implicated sequence polymorphism in antigenic escape (Healer *et al.* 2004), and cross-strain inhibition assays suggested that the extent of escape correlated with sequence distance between the vaccine and the target strain (Kennedy *et al.* 2002).

In an attempt to overcome strain specificity to potential vaccine candidate AMA-1, vaccine researchers combined allelic variants of this antigen and immunized rhesus monkeys with a mixture of two allelic forms of PfAMA-1 (designated AMA-1-C1) or with a single AMA-1 allele and measured responses *in vitro* using GIA and ELISA (Kennedy *et al.* 2002). The resulting antibodies were similarly effective regardless of an immunization with a single variant or the mixture AMA-1-C1. Moreover, phase 1 studies in malaria naïve adults in the

US, and semi-immune adults and children in Mali showed that, the vaccine AMA-1-C1 is safe and immunogenic, although in children the duration of the induced antibody was short (Malkin *et al.* 2005, Dicko *et al.* 2007 & 2008). When Lalitha and co-workers (2008) induced mice and rabbits with two allelic forms of domain I and II of PfAMA-1, results showed that both the AMA-1 domains I and II allelic variants either in combination or each by itself could induce significant immune responses in mice and rabbits in combination with Freund's adjuvant. On the contrary, a study conducted by Chessman and colleagues (2009) demonstrated that, strains with the lowest sequence identity at MSP-1_{21} in *P. chabaudi* (corresponding to MSP-1_{19} of *P. falciparum*) induced robust and reciprocal strain-specific protective immunity (SSPI) in experimental mice, while two strains that did not induce reciprocal SSPI were identical at the 21 kDa region. Therefore, these contradictory results raise questions as to the necessity of using multi-allele vaccines.

5.1.6. Objective of the current study

As a multi-component malaria vaccine containing different antigens would be the final goal of vaccine development to combat malaria (Pan *et al.* 2004). The Merozoite Surface Protein-1 in the form of MSP-1_{42} and MSP-1_{19}, and the Apical Membrane Antigen-1 are potential candidates for future vaccine trials against *P. vivax* malaria. The natural antibody response against PvAMA-1, PvMSP-1_{42} and PvMSP-1_{19} (Wickramarachchi *et al.* 2006 & 2007) was demonstrated previously where particularly PvAMA-1 and PvMSP-1_{19} reflected a protective IgG dominated immune response with increasing exposure in malaria endemic residents. However, 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 polymorphism is a crucial factor incorporated by the parasite to escape the host immune system, the objective of the present study was to assess the possible strain-specific or strain-transcending immune responses generated against PvAMA-1 domain II, PvMSP-1_{42} and PvMSP-1_{19}.

5.2. Research design

The present cross sectional immuno-epidemiological study was designed to characterize the natural 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 pronged into two and 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-1_{42} and PvMSP-1_{19} 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.

Thirty nine amino acid polymorphic sites on PvMSP-1_{42} generated 27 amino acid haplotypes, where all the polymorphism was clustered towards the 33 kDa region, while the 19 kDa was identical in all 95 *P. vivax* clinical isolates (chapter 4). To evaluate possible strain-specific immune responses against PvMSP-1_{42}, seventy two of the 95 *P. vivax* MSP-1_{42} amino acid sequences, representing 25 of the 27 amino acid haplotypes were aligned with the homologous total (IgM + IgG) anti-PvMSP-1_{42} antibody responses previously assayed by an indirect ELISA against recombinant PvMSP-1_{42} protein (Wickramarachchi *et al.* 2007). The recombinant protein PvMSP-1_{42} expressed in the baculovirus system resembled the native Belem construct (Longacre *et al.* 1994; Holm *et al.* 1997). This analysis was repeated for PvMSP-1_{19}.

5.3. Results

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

5.3.1.1. Total (IgM + IgG) antibody responses against synthetic peptides representing 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 were designed with overlapping 4-6 amino acids (Figure 5.1). Peptide specific total (IgM + IgG) antibody responses were assayed using an indirect micro plate ELISA in 162 serum samples exposed to malaria 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 an exposure to malaria (normal healthy controls) (Appendix XIII). The 162 serum samples were positive for total anti-AMA-1 antibodies assayed against the recombinant PvAMA-1 protein (PV66/AMA-1) as recorded previously (Wickramarachchi, 2005).

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

Figure 5.2 (A-G scatter plots) and Table 5.1 present the total antibody responses to PvAMA-1 domain II peptides P01 to P07, of individuals from 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 5.1).

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 5.1).

5.3.1.1.2. Comparison of antibody prevalence against PvAMA-1 domain II peptides among the three test areas

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 5.1, 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 5.1, Chi square test, $P>0.05$). No significant difference in antibody prevalence to each of these seven peptides was evident between the acute PNE and PE patients from Colombo (Chi square test, $P>0.05$, Table 5.1).

A significantly high prevalence of non-responders in all three test areas was recorded against P02, P03 and P06 (Table 5.2). While, the responding prevalence to P05 was significantly higher than the non-responders in both endemic areas, the prevalence of non-responders to P05 were significantly higher in non-endemic Colombo. No significant difference in responding and non-responding prevalence was detected in individuals from Anuradhapura against P01, P04 and P07. However, the non-responders against P01 and P07 were significantly higher than the responders in Kataragama and Colombo. Conversely, the responders were significantly higher in both Kataragama and Colombo against P04 (Table 5.2). Though, both the exposure groups in Colombo recorded a significantly high prevalence of non-responders than responders against P03, P05, P06 and P07, only individuals from the PE group to P01 and P02 recorded a significantly high prevalence of non-responders than responders while individuals from the PNE group to P04 recorded the same trend (Table 5.2).

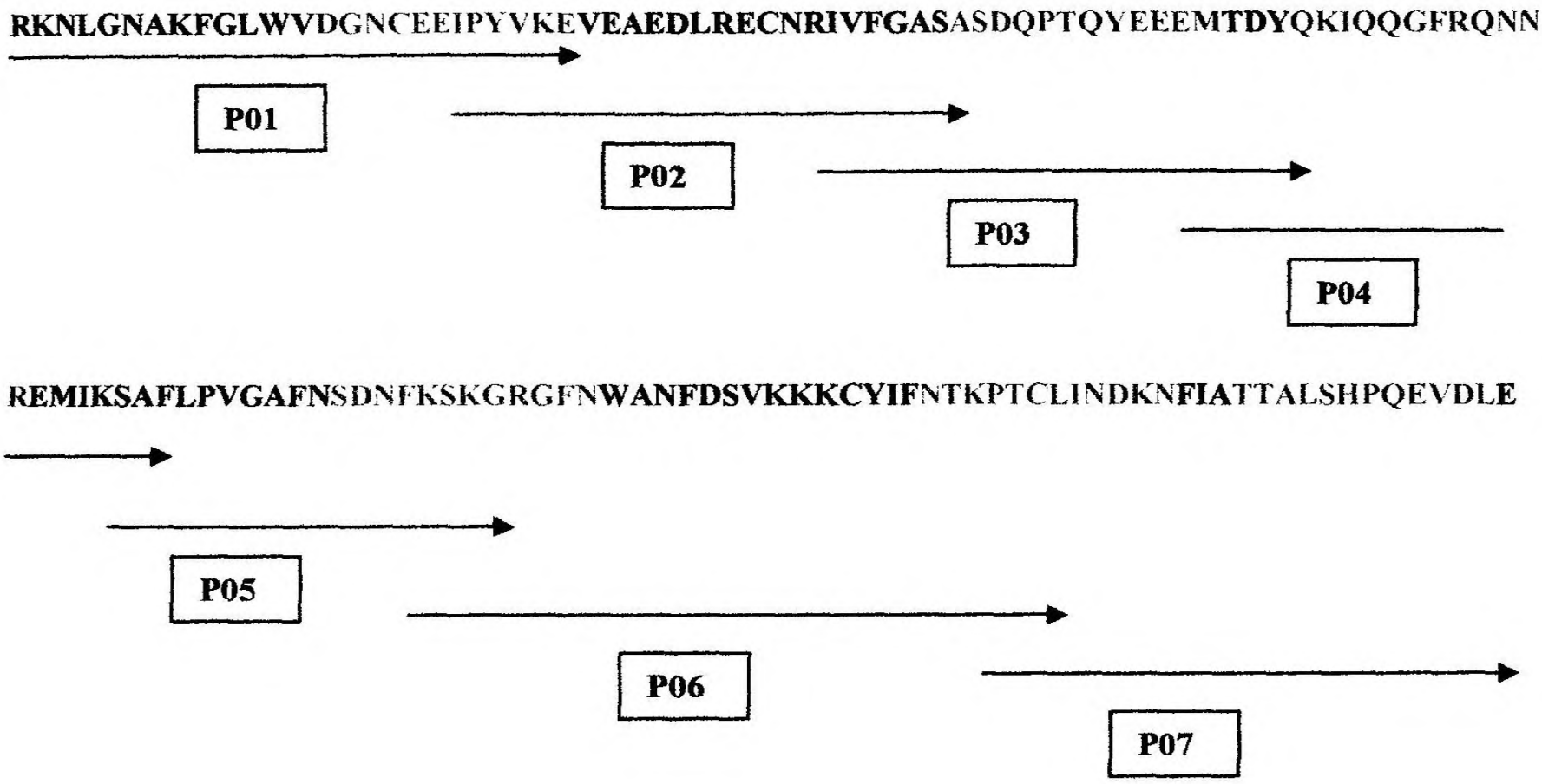
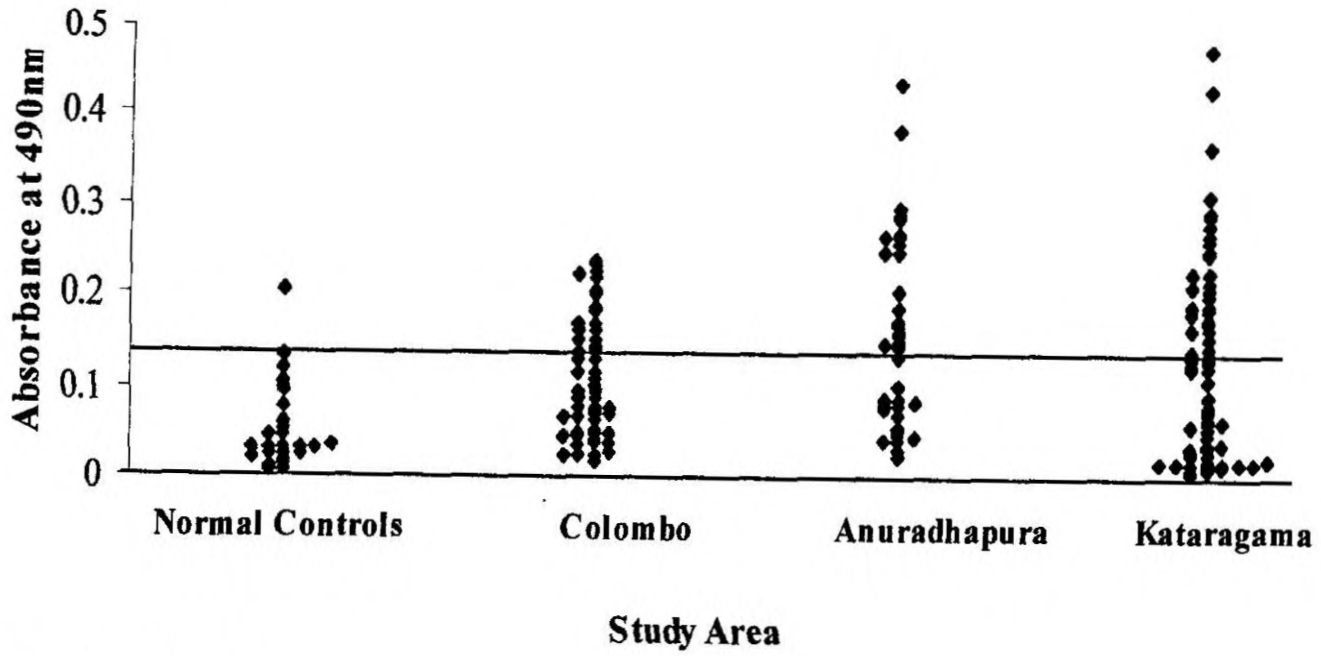


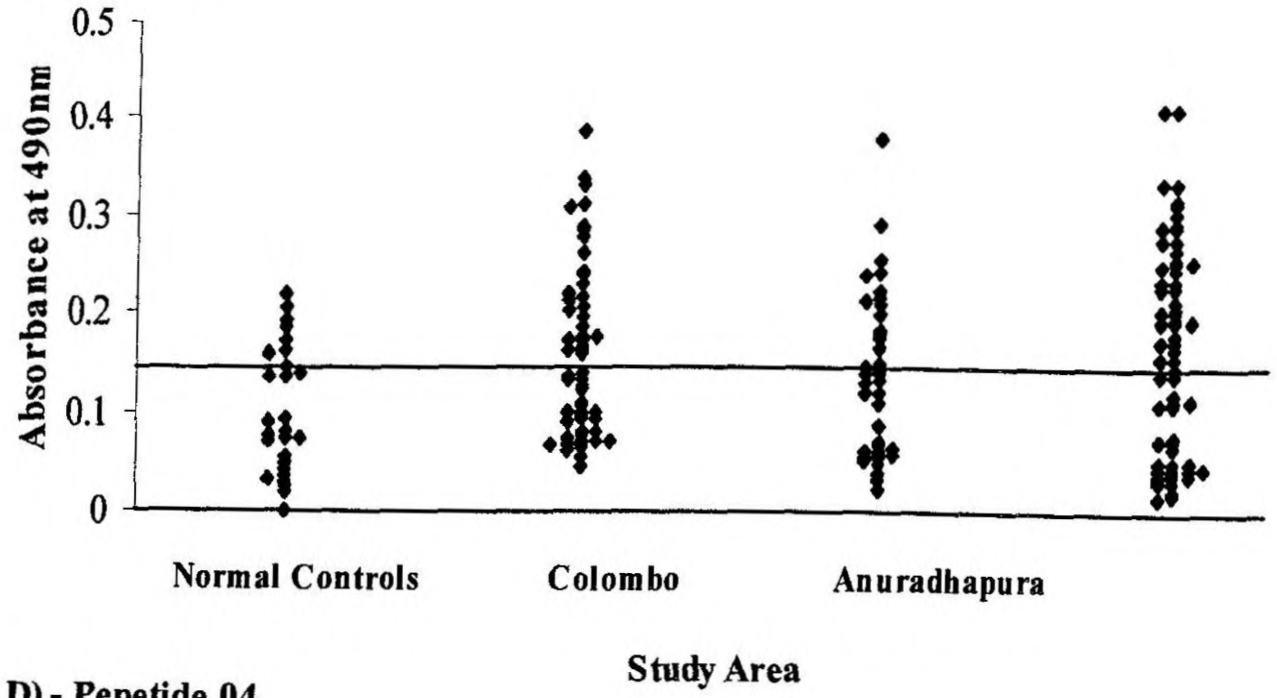
Figure 5.1. Schematic representation of the seven synthetic peptides designed to PvAMA-1 domain II

Predicted linear B cell epitopes are depicted in blue.

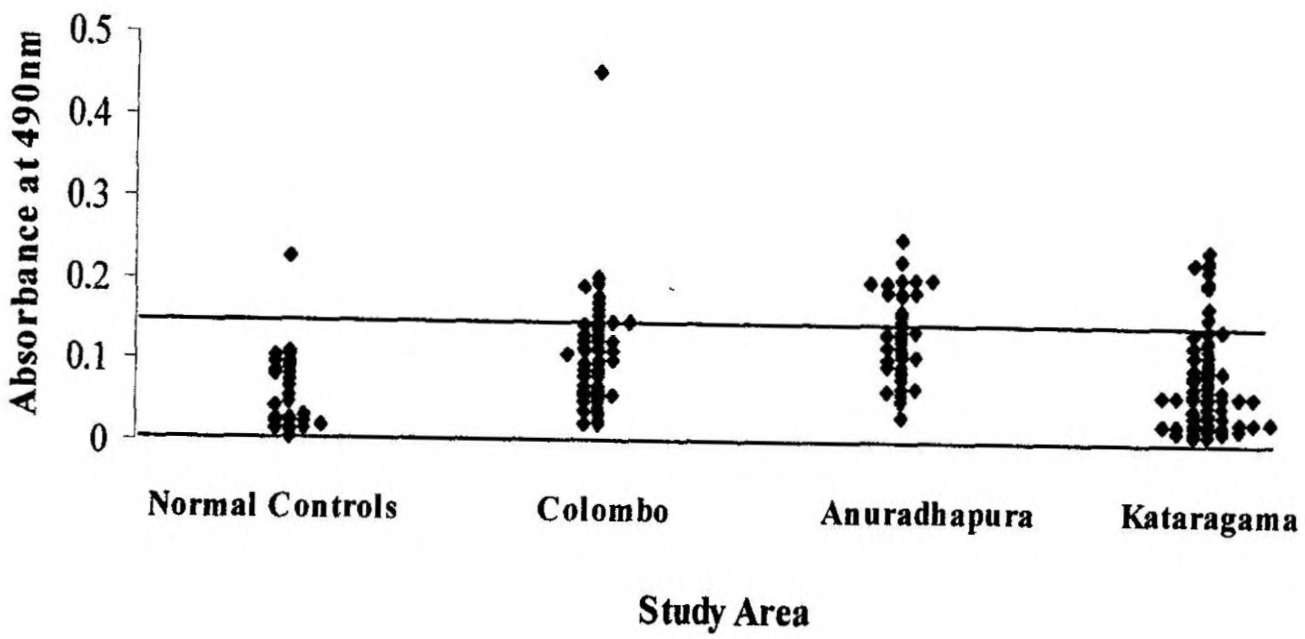
A) - Pepetide 01



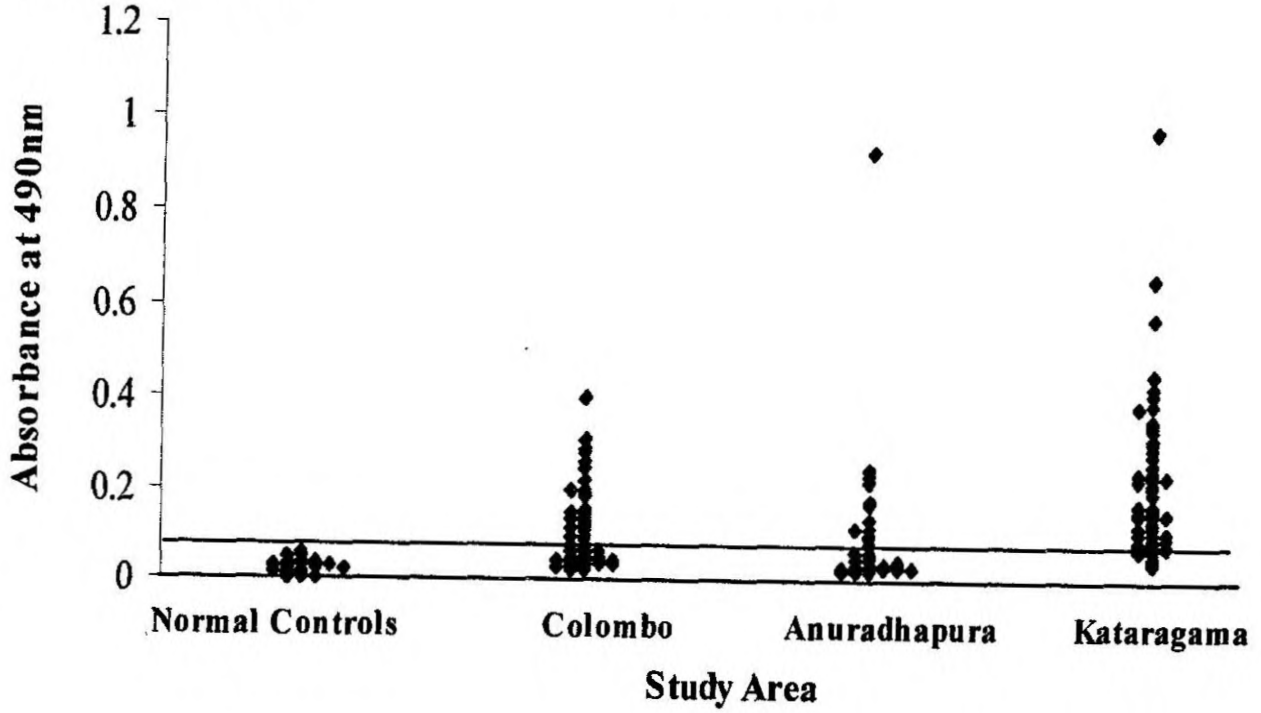
B) - Pepetide 02

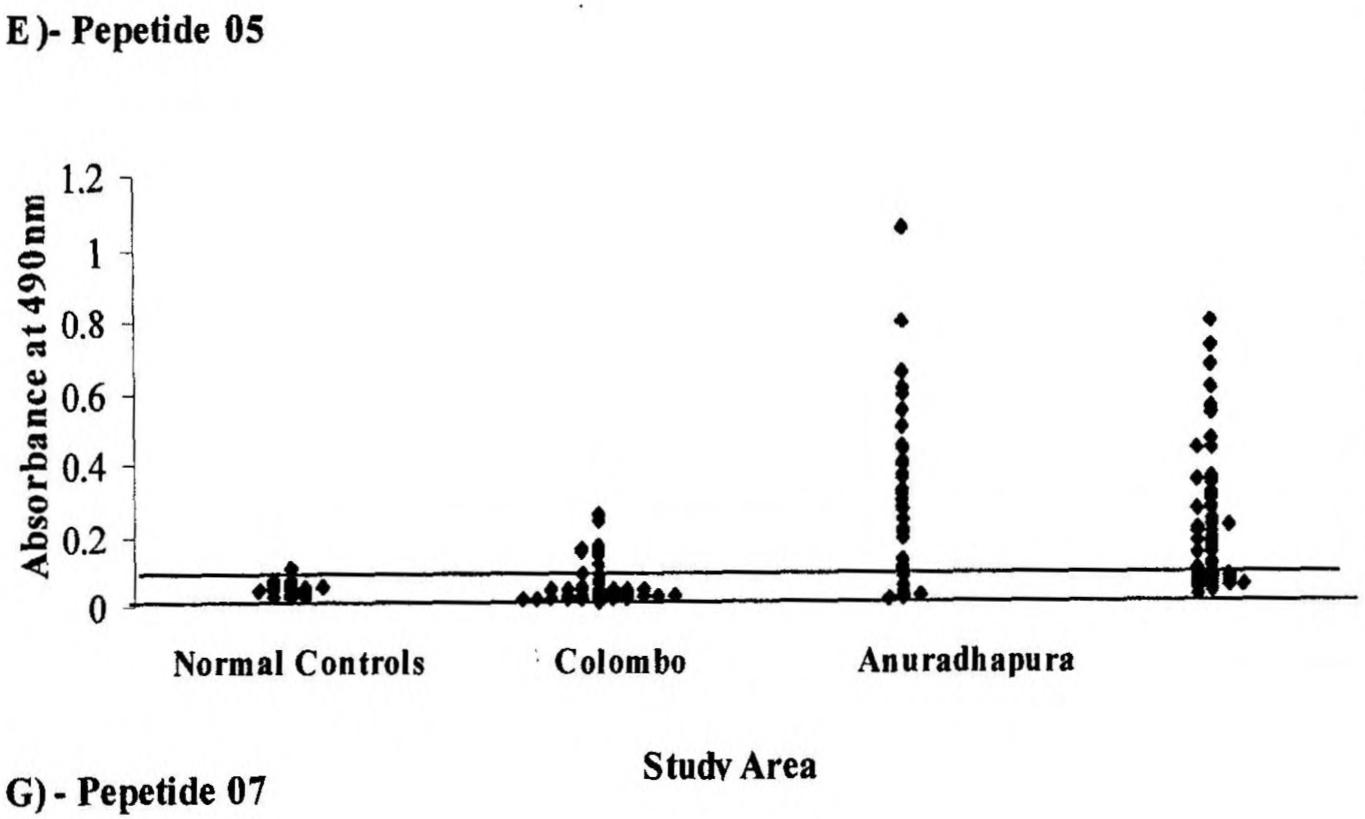


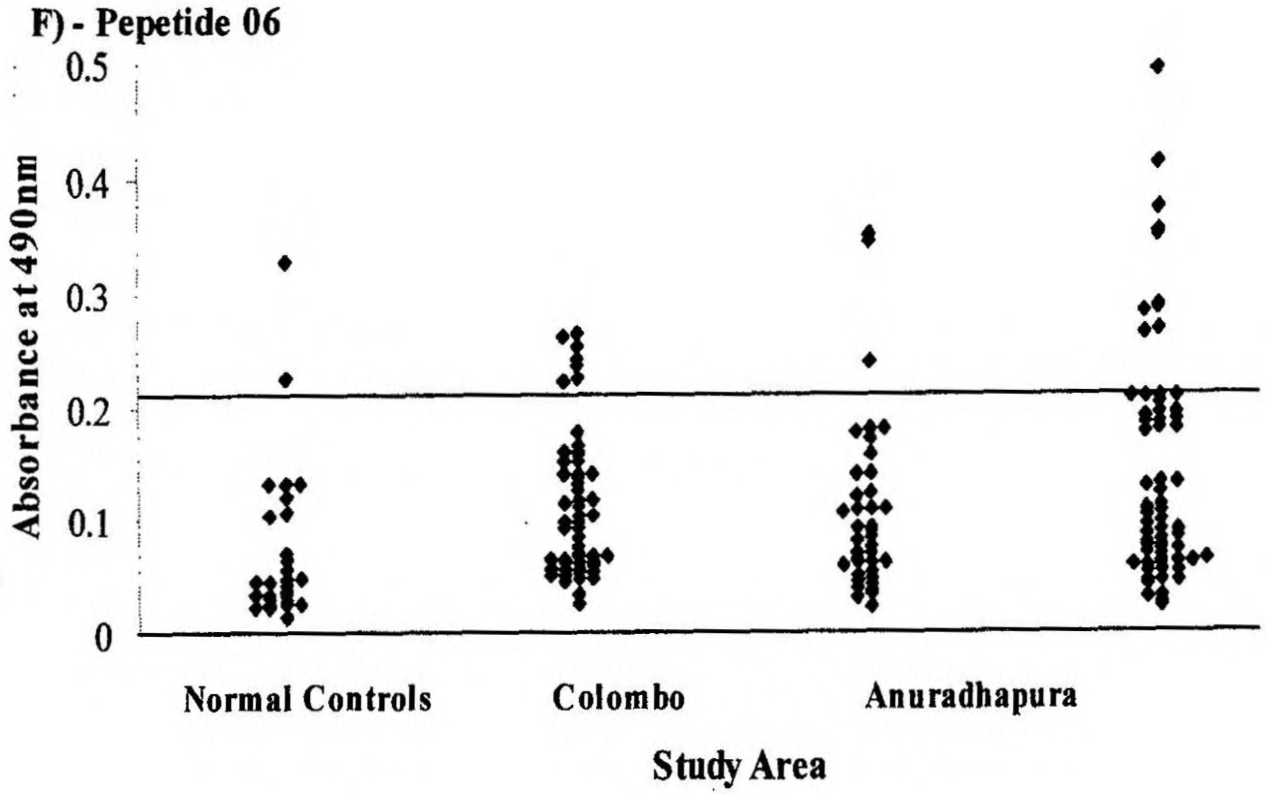
C) - Pepetide 03



D) - Pepetide 04







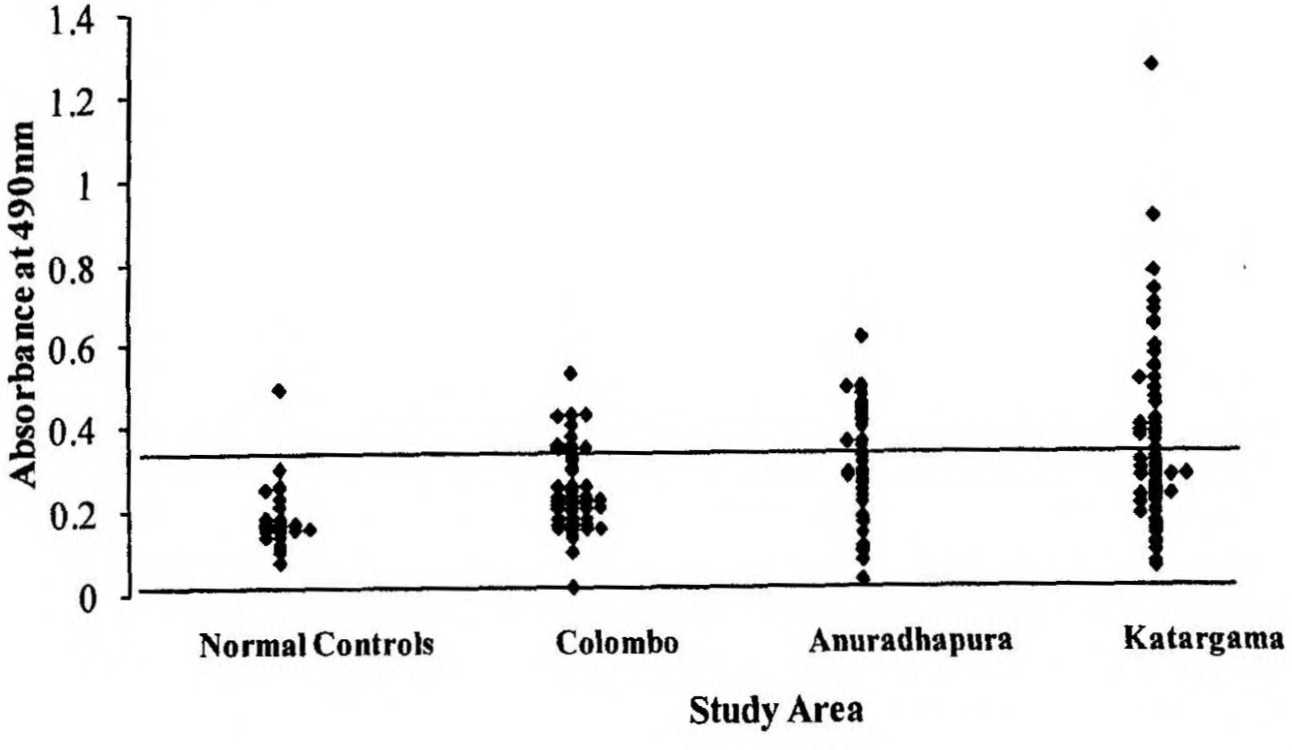


Figure 5.2. Anti-PvAMA-1 antibody response against domain II peptides (A) P01 (B) P02 (C) P03 (D) P04 (E) P05 (F) P06 (G) P07 in the three study areas

Each closed circle represents the mean of duplicate absorbance values at 490 nm, of each serum sample. The numbers of individuals in the normal control group and the test groups from Colombo, Anuradhapura and Kataragama were 28, 45, 32 and 55, respectively. Horizontal line indicates the cut off value (the mean absorbance value at 490 nm plus 2 standard deviation of the normal healthy individuals from Colombo with no past history of malaria). Mean OD values obtained for test samples falling over and above this cut-off level were expressed as positive responses.

Table 5.1. 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
Anuradhapura	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 (patients suffering their first malaria infection)

Table 5.2. Prevalence of responders and non-responders to anti-PvAMA-1 antibodies against P01, P02, P03, P04, P05, P06 and P07 in acute *P. vivax* patients from the three study areas

Study area (Number of samples)	Peptide 1				Peptide 2				Peptide 3				Peptide 4			
	Prevalence (%)		Chi-Square value	P-value	Prevalence (%)		Chi-Square value	P-value	Prevalence (%)		Chi-Square value	P-value	Prevalence (%)		Chi-Square value	P-value
	Responders	Non Responders			Responders	Non Responders			Responders	Non Responders			Responders	Non Responders		
Colombo (Total) (N=56)	28.6 (N=16)	71.4 (N=40)	18.89	0.000***	21.4 (N=12)	78.6 (N=44)	34.32	0.000***	12.5 (N=7)	87.5 (N=49)	60.04	0.000***	64.3 (N=36)	35.7 (N=20)	8.04	0.0045***
PE [a] (N=36)	19.4 (N=7)	80.6 (N=29)	24.5	0.000***	13.9 (N=5)	86.1 (N=31)	34.72	0.000***	5.6 (N=2)	94.4 (N=34)	53.39	0.000***	61.1 (N=22)	38.9 (N=14)	2.72	0.0989
PNE [b] (N=15)	40 (N=6)	60 (N=9)	0.53	0.456	33.3 (N=5)	66.7 (N=10)	2.13	0.144	13.3 (N=2)	86.7 (N=13)	13.33	0.000***	73.3 (N=11)	26.7 (N=4)	4.8	0.028*
Anuradapura (N=39)	46.2 (N=18)	53.8 (N=21)	0.21	0.650	12.8 (N=5)	87.2 (N=34)	40.21	0.000***	33.3 (N=13)	66.7 (N=26)	7.38	0.006**	42.1 (N=16)	57.9 (N=22)	1.32	0.251
Kataragama (N=67)	40.3 (N=27)	59.7 (N=40)	4.3	0.038*	31.3 (N=21)	68.7 (N=46)	17.19	0.000***	13.4 (N=9)	86.6 (N=58)	68.78	0.000***	95.4 (N=62)	4.6 (N=3)	103.51	0.000***

Study area (Number of samples)	Peptide 5				Peptide 6				Peptide 7			
	Prevalence (%)		Chi-Square value	P-value	Prevalence (%)		Chi-Square value	P-value	Prevalence (%)		Chi-Square value	P-value
	Responders	Non Responders			Responders	Non Responders			Responders	Non Responders		
Colombo (Total) (N=56)	25 (N=14)	75 (N=42)	26.04	0.000***	12.5 (N=7)	87.5 (N=49)	60.04	0.000***	19.6 (N=11)	80.4 (N=45)	38.89	0.000***
PE [a] (N=36)	19.4 (N=7)	80.6 (N=29)	24.5	0.000***	11.1 (N=4)	88.9 (N=32)	40.5	0.000***	16.7 (N=6)	83.3 (N=30)	29.39	0.000***
PNE[b] (N=15)	26.7 (N=4)	73.3 (N=11)	4.8	0.028*	6.7 (N=1)	93.3 (N=14)	19.2	0.000***	26.7 (N=4)	73.3 (N=11)	4.8	0.000***
Anuradapura (N=39)	73.7 (N=28)	26.3 (N=10)	15.21	0.000***	7.7 (N=3)	92.3 (N=36)	52.51	0.000***	43.6 (N=17)	56.4 (N=22)	0.82	0.365
Kataragama (N=67)	69.7 (N=46)	30.3 (N=20)	18.94	0.000***	14.9 (N=10)	85.1 (N=57)	63.16	0.000***	38.8 (N=26)	61.2 (N=41)	5.85	0.0155*

[a] – previously exposed to malaria

[b] – previously not exposed to malaria

*- at a significant level of P<0.05

**- at a significant level of P<0.01

***- at a significant level of P<0.001

5.3.1.1.3. 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 5.3).

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 5.3). 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 5.3).

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 5.1). 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 5.3).

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 Anuradhapura and Kataragama and for P01/P05 exclusively from Anuradhapura (Table 5.3). 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 5.3).

5.3.1.1.4. 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 (Kruskal-Wallis test, $P<0.05$, Table 5.4). A significantly high antibody magnitude was recorded from Anuradhapura compared to Kataragama and Colombo against P05 (Kruskal-Wallis test, $P<0.05$, Table 5.4). 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 5.4). 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, Figure 5.3Ai) and P04/P07 (r = 0.886, Figure 5.3Aii) among patients from non-endemic Colombo, ($P<0.05$) and endemic Kataragama (P04/P05, r = 0.36, P04/P07, r = 0.474, $P<0.01$, Figure 5.3Ci and ii), while, a negative correlation was observed among responders of Anuradhapura against peptides P04/P05 (r = -0.734, $P<0.05$, Figure 5.3B). A significant positive correlation between antibody magnitudes against P02/P07 was exclusively observed from Kataragama (r = 0.509, $P<0.05$, Figure 5.3Ciii).

Table 5.3. 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

Table 5.4. 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.

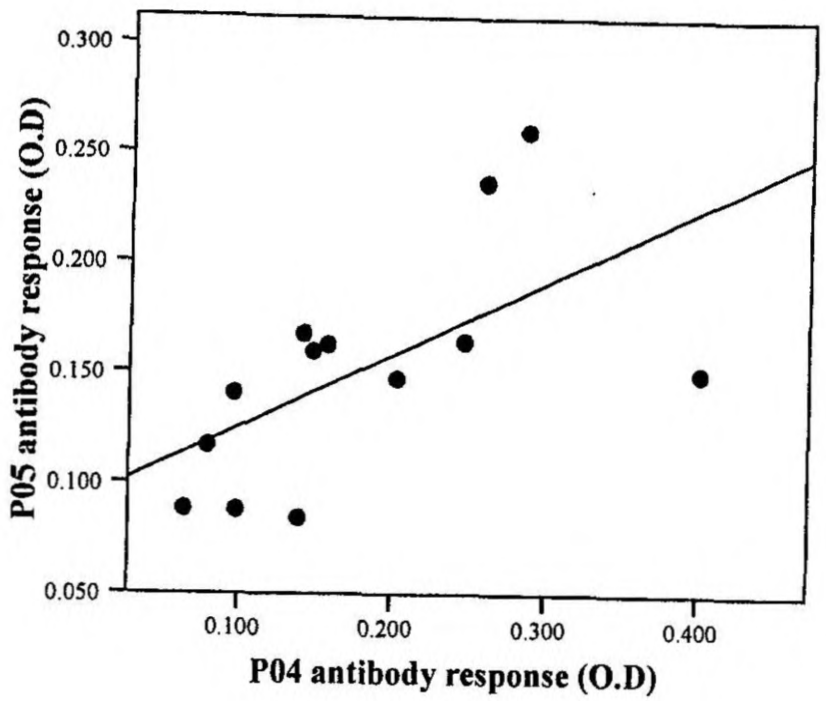
[b] – Standard Error of Mean

[c] – Previously exposed to malaria

[d] – Previously not exposed to malaria

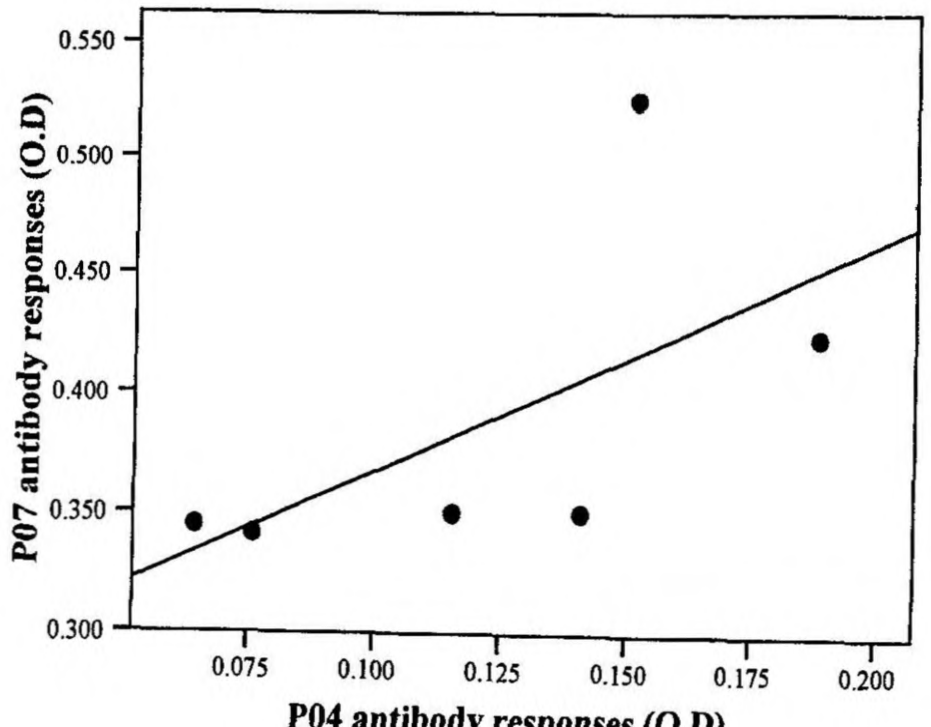
A) Colombo

i



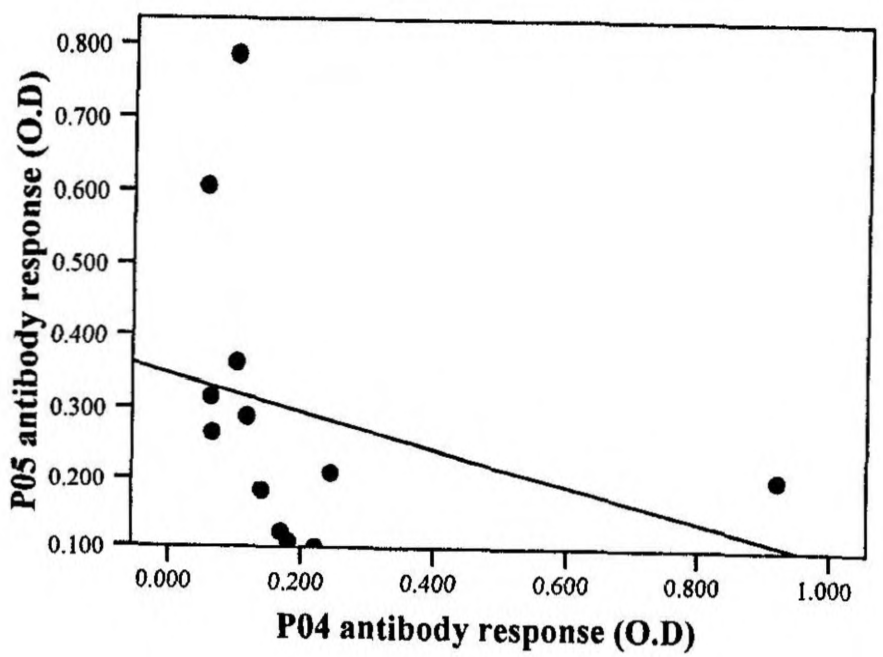
Spearman correlation coe
(r) = 0.709
P = 0.007

ii



Spearman correlation coefficient
(r) = 0.886
P = 0.019

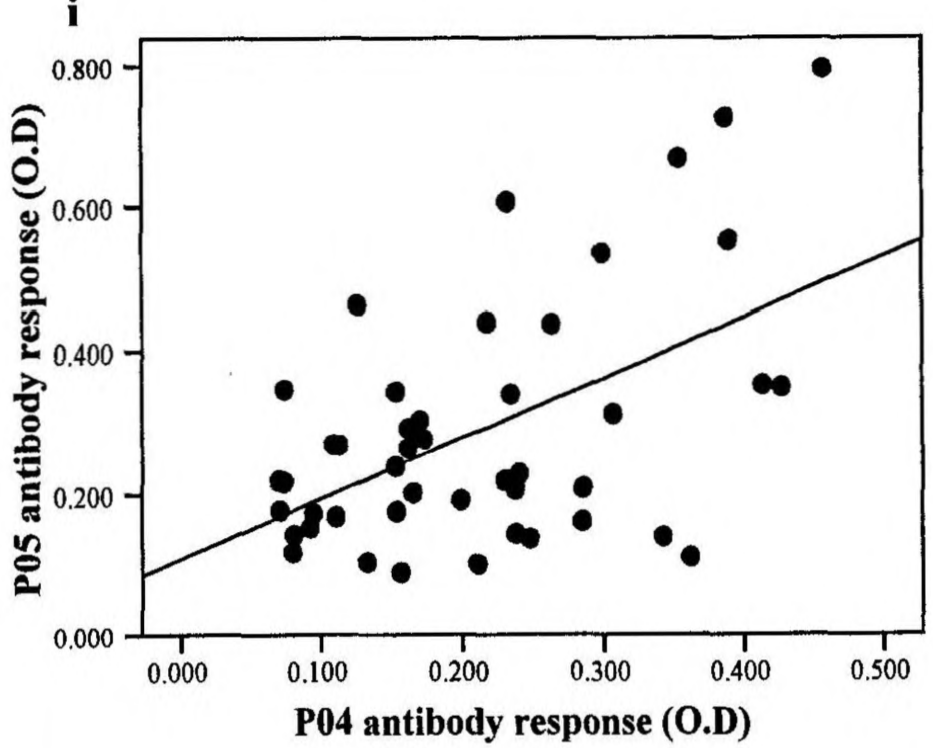
B) Anuradhapura



Spearman correlation coefficient
(r) = -0.739
P = 0.007

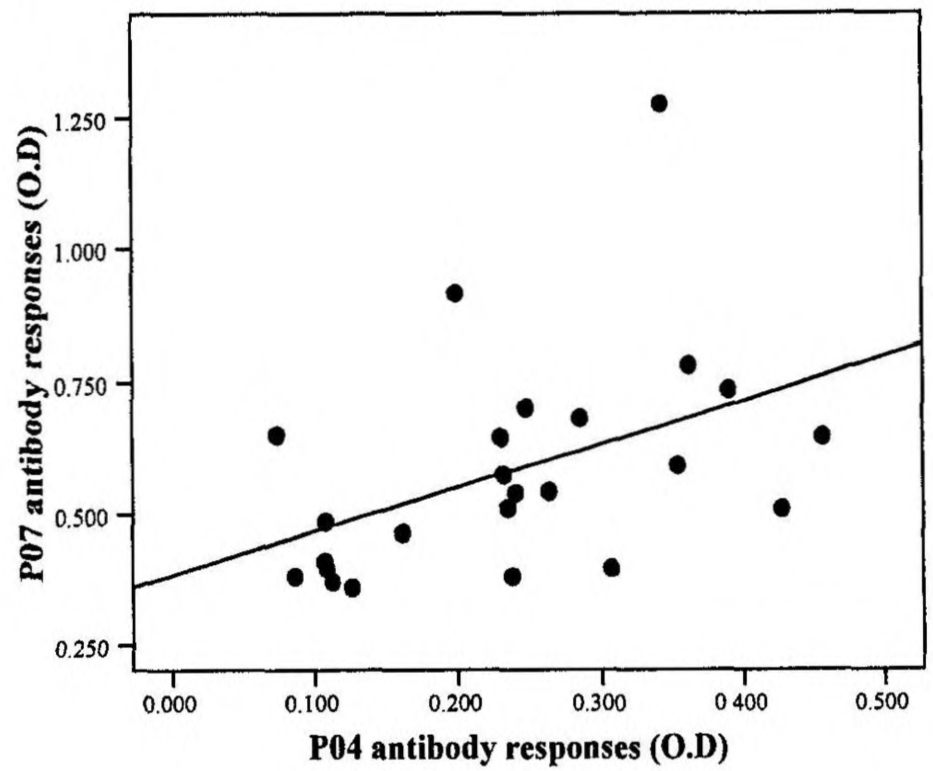
C) Kataragama

i



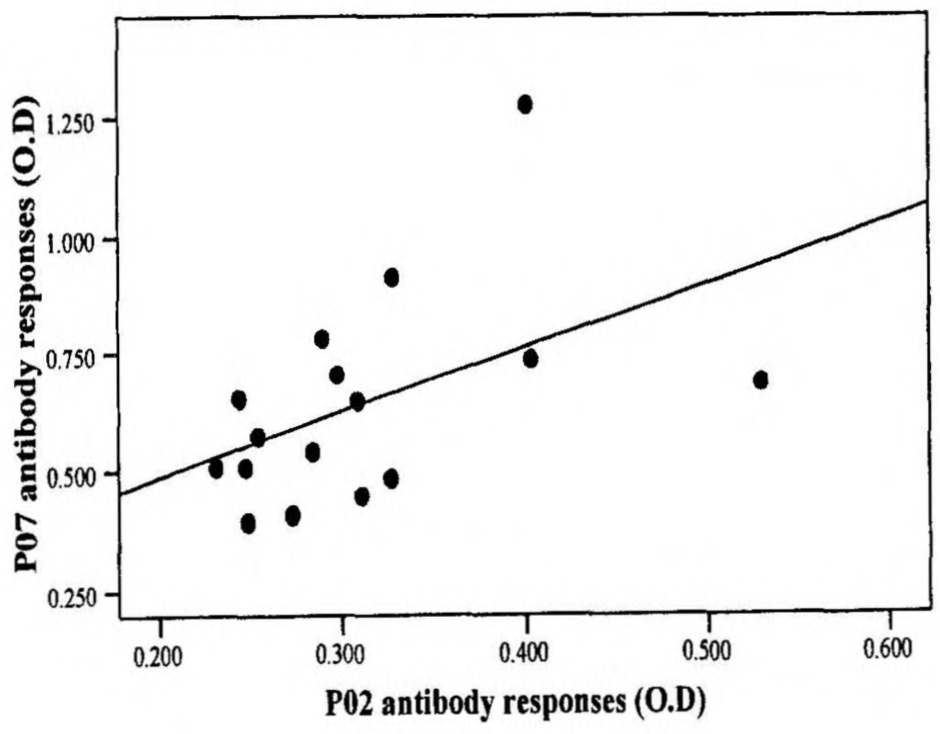
Spearman correlation coefficient
(r) = 0.36
P = 0.015

ii



Spearman correlation coefficie
(r) = 0.474
P = 0.019

iii



Spearman correlation coefficient
(r) = 0.509
P = 0.044

Figure 5.3. Correlation of total (IgM+IgG) anti-PvAMA-1 antibod magnitudes of each individual from the three study areas

(A) correlation among Colombo responders to i. P04/P05, ii. P04/P07

(B) correlation among Anuradhapura responders to P04/P05

(C) correlation among Kataragama responders to i. P04/P05, ii. P04/P07 and iii. P02/P07

The trend line, spearman correlation coefficient value and the P value ar depicted in each figure. Each closed circle represents an individual th obtained a positive mean value of an antibody magnitude against tw selected peptides.

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

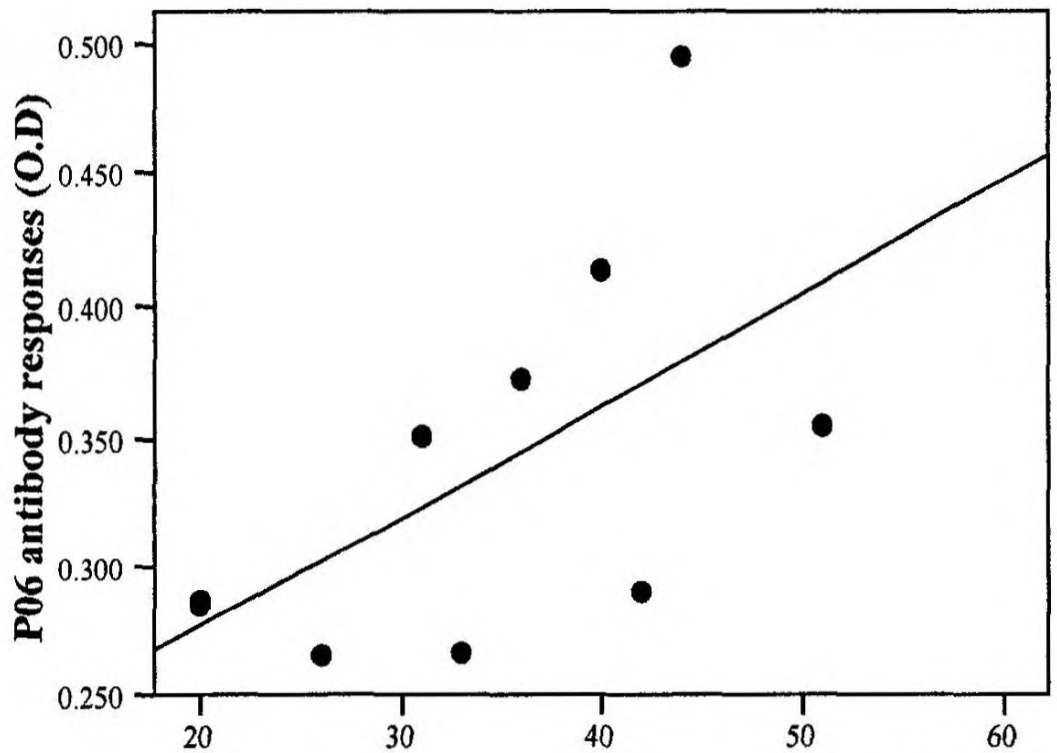
Attempts were made to establish associations between total (IgM + IgG) antibody magnitudes and responding prevalence with different host factors (*i.e.* age of patient, parasitaemia and the number of past infections) using bivariate Spearman correlation tests and Chi square test for linear trend.

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 ($P<0.05$, Figure 5.4A and B).

A significant positive correlation was evident between previous exposure with antibody magnitudes against P05 in patients from Anuradhapura (Spearman correlation coefficient = 0.532, $P<0.01$, Figure 5.4C). Majority of the responders against P05 were those who either lacked previous exposure to the infection, or who had an exposure of 1-2 infections only.

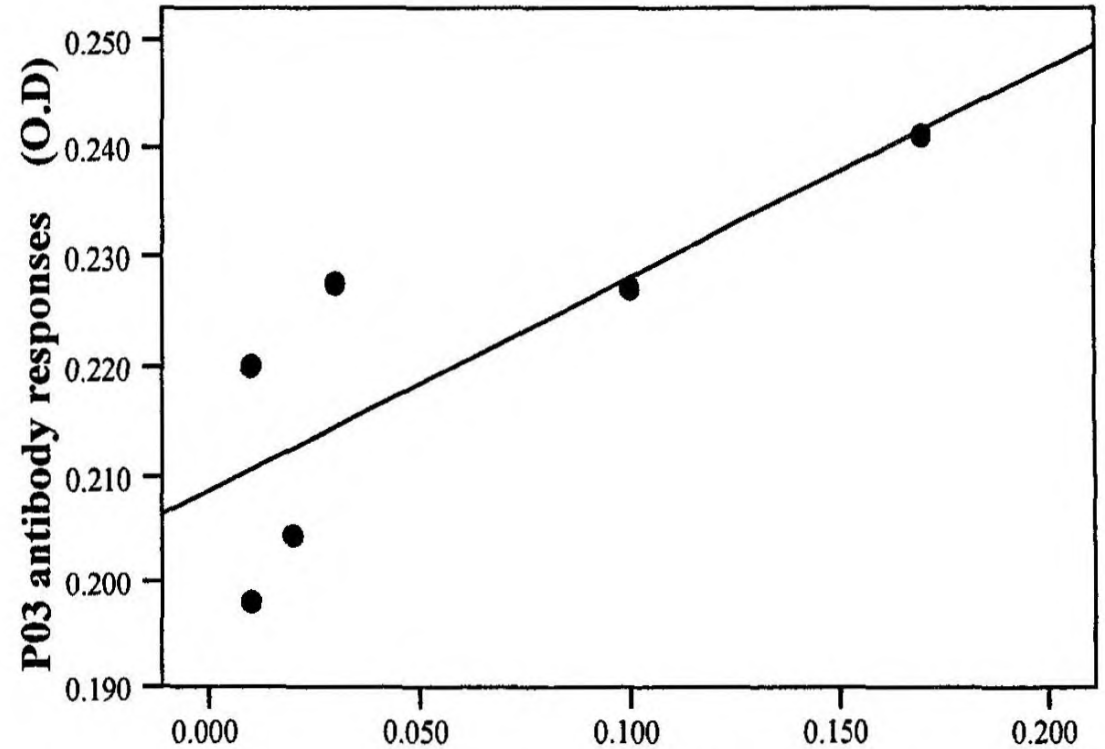
For further analysis of associations between the responding proportions and exposure groups, the number of past infections within each study area was grouped into i) 0 ii) 1-2 iii) 3-5 and iv) >5 past infections. A positive trend was evident between the number of past infection groups of Kataragama and the responding prevalence against peptides P01, P02, P04, P05 and P07 (Chi square for linear trend, $P>0.05$) (Figure 5.5).

A) - Kataragama



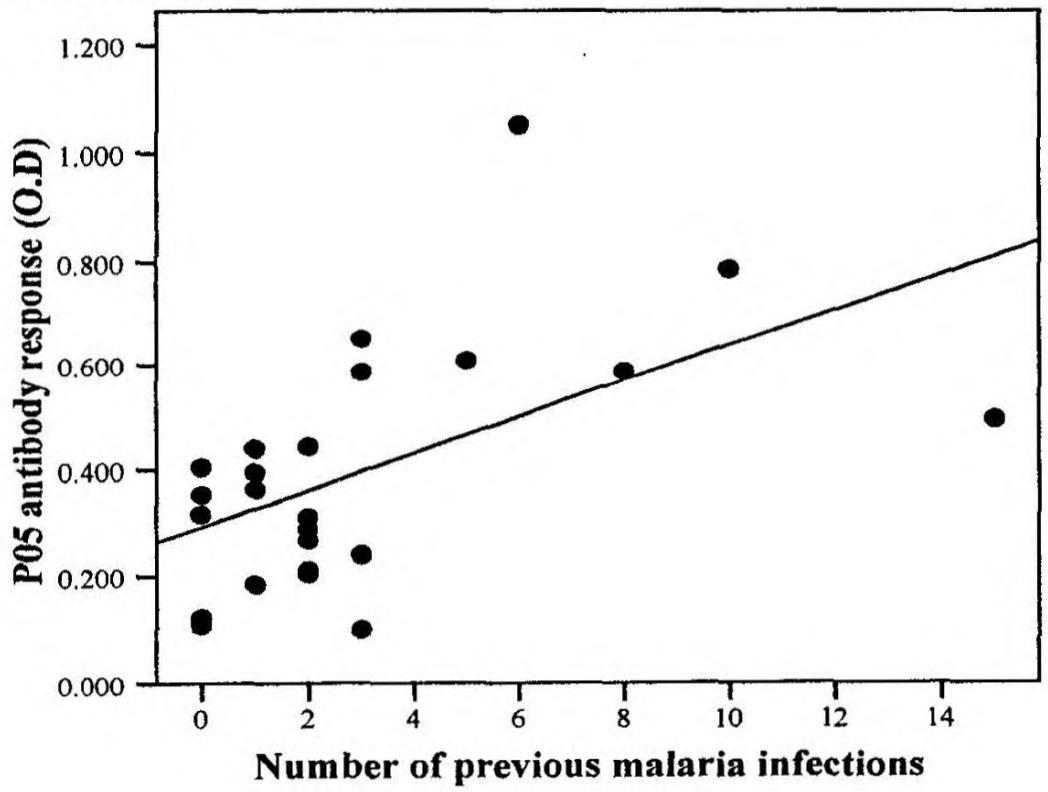
Spearman correlation coefficient (r) = 0.681
P = 0.03

B)- Kataragama ▲



Spearman correlati
coefficient (r) = 0.8
P = 0.019

C) - Anuradhapura



Spearman correlation coefficient (r) = 0.532
P = 0.007

Figure 5.4. Association between total (IgM + IgG) PvAMA-1 domain II peptide antibody responses with host factors

Each closed circle represents the mean absorbance of a serum sample from a single individual.

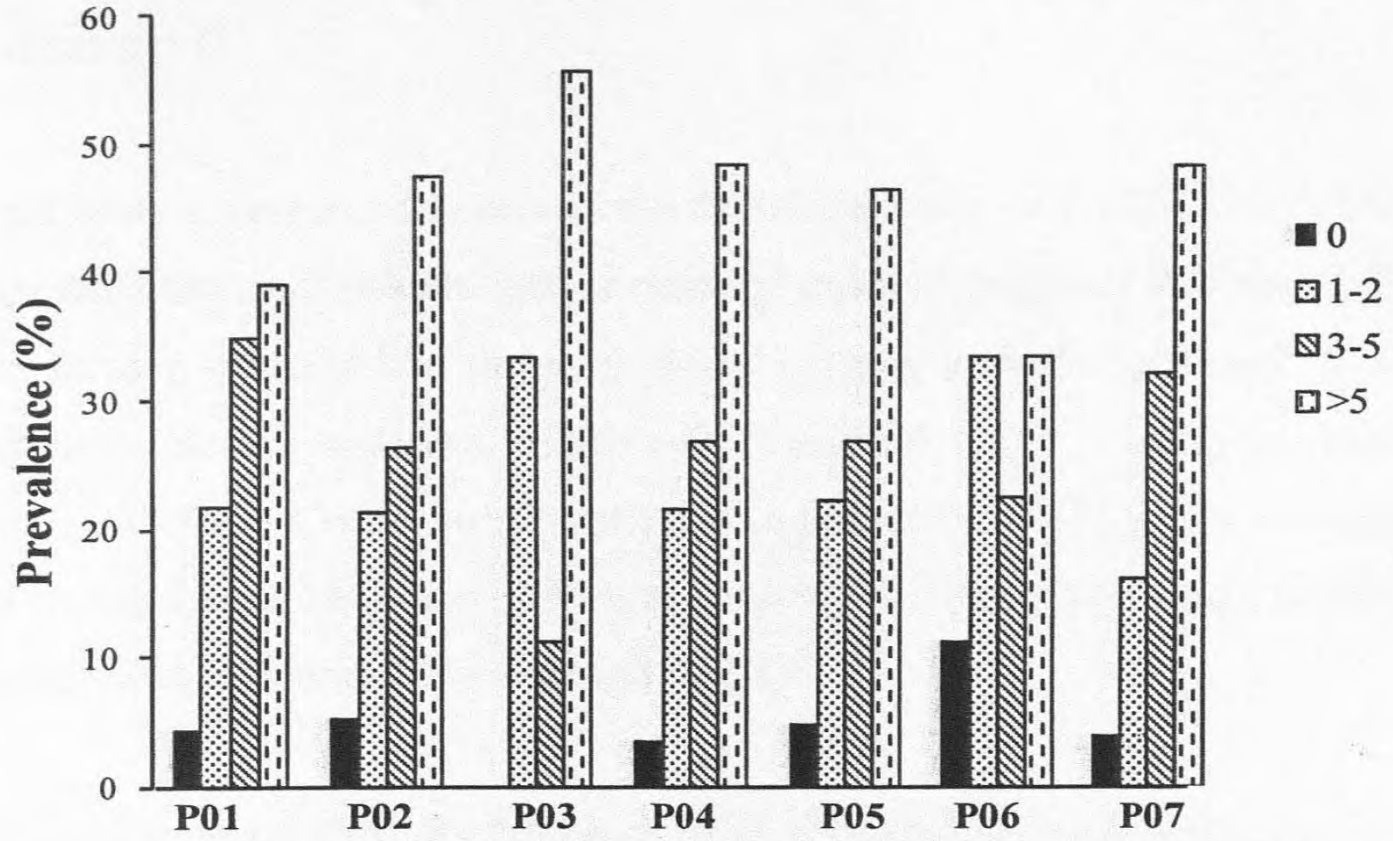


Figure 5.5. Antibody prevalence against the synthetic peptides of PvAMA-1 domain II with different malaria exposure groups in Kataragama

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

5.3.1.2. 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 (Appendix XIII).

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

Figure 5.6 (A and B scatter plots) and Table 5.5 present the IgM antibody response to PvAMA-1 P04 and P05 of individuals from the three study areas. 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 5.5). Lack of anti-PvAMA-1 IgM antibody response was observed in the PNE group for P04 and in the PE group for P05.

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

Figure 5.6 (C and D scatter plots) and Table 5.5 present the IgG antibody response to PvAMA-1 P04 and P05 of individuals from the three study areas. 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 5.5).

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

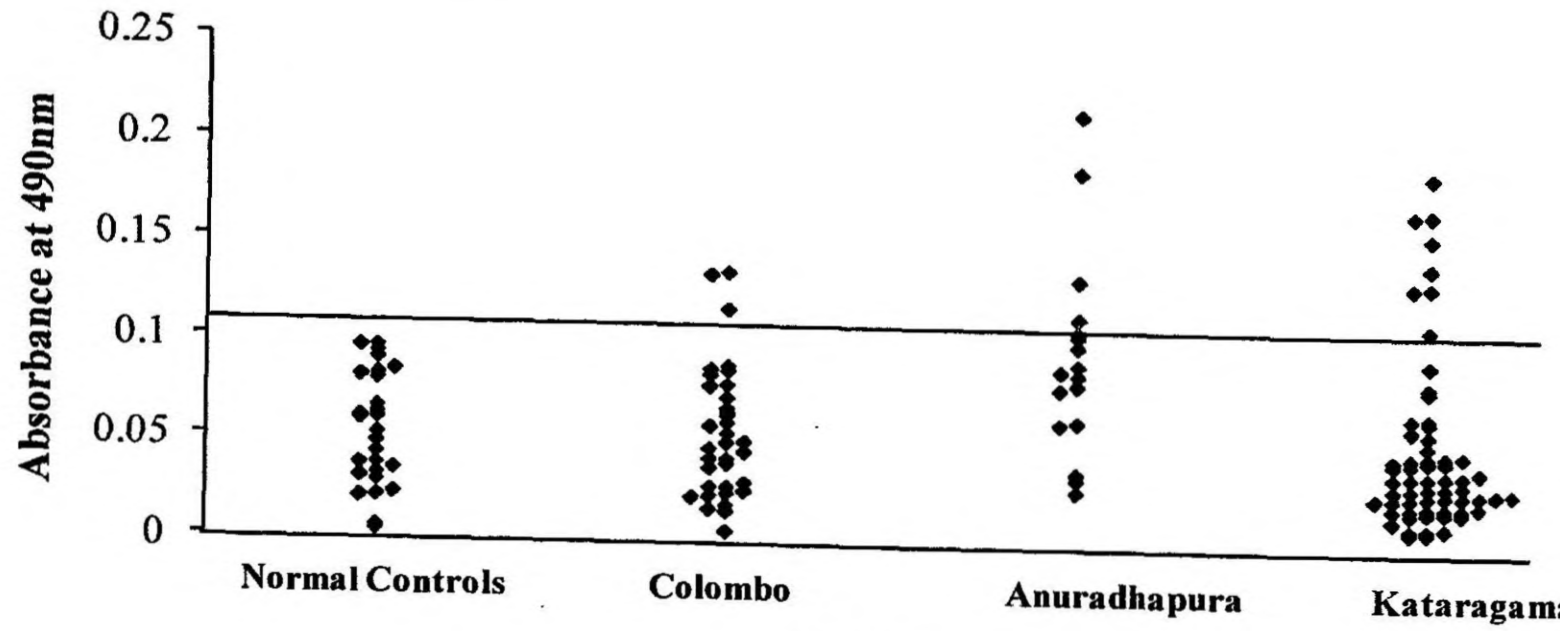
Individuals from Anuradhapura recorded significantly elevated IgM antibody prevalence against P05 compared to P04, conversely, individuals from Kataragama recorded significantly elevated IgG antibody prevalence against P04 compared to P05 (Chi square test, $P<0.05$, Table 5.5). Though, patients from 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 5.5).

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

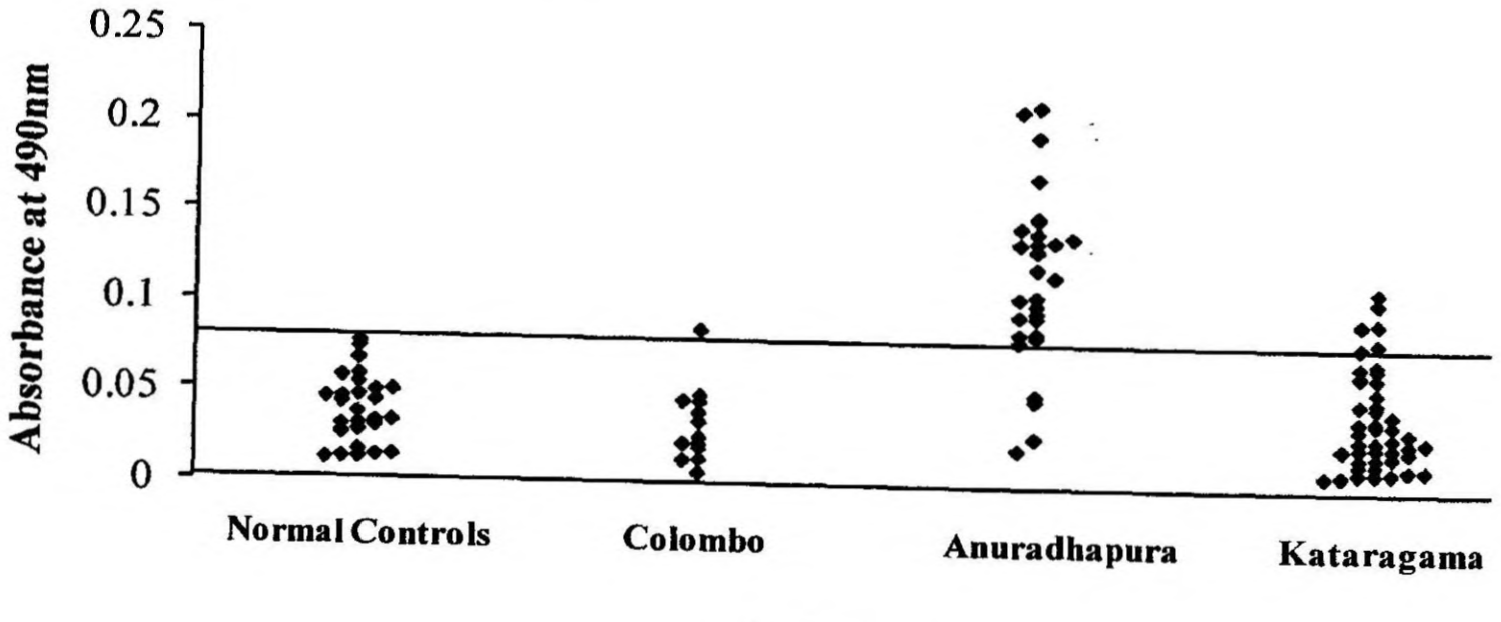
The IgM isotype antibody magnitude against P05 was significantly lower compared to P04 in all three study areas (Mann Whitney U test, $P<0.05$, Table 5.6). While an elevated IgG antibody magnitude was recorded from Anuradhapura against P05 compared to P04, the other two study areas recorded the reverse (Mann Whitney U test, $P>0.05$, Table 5.6).

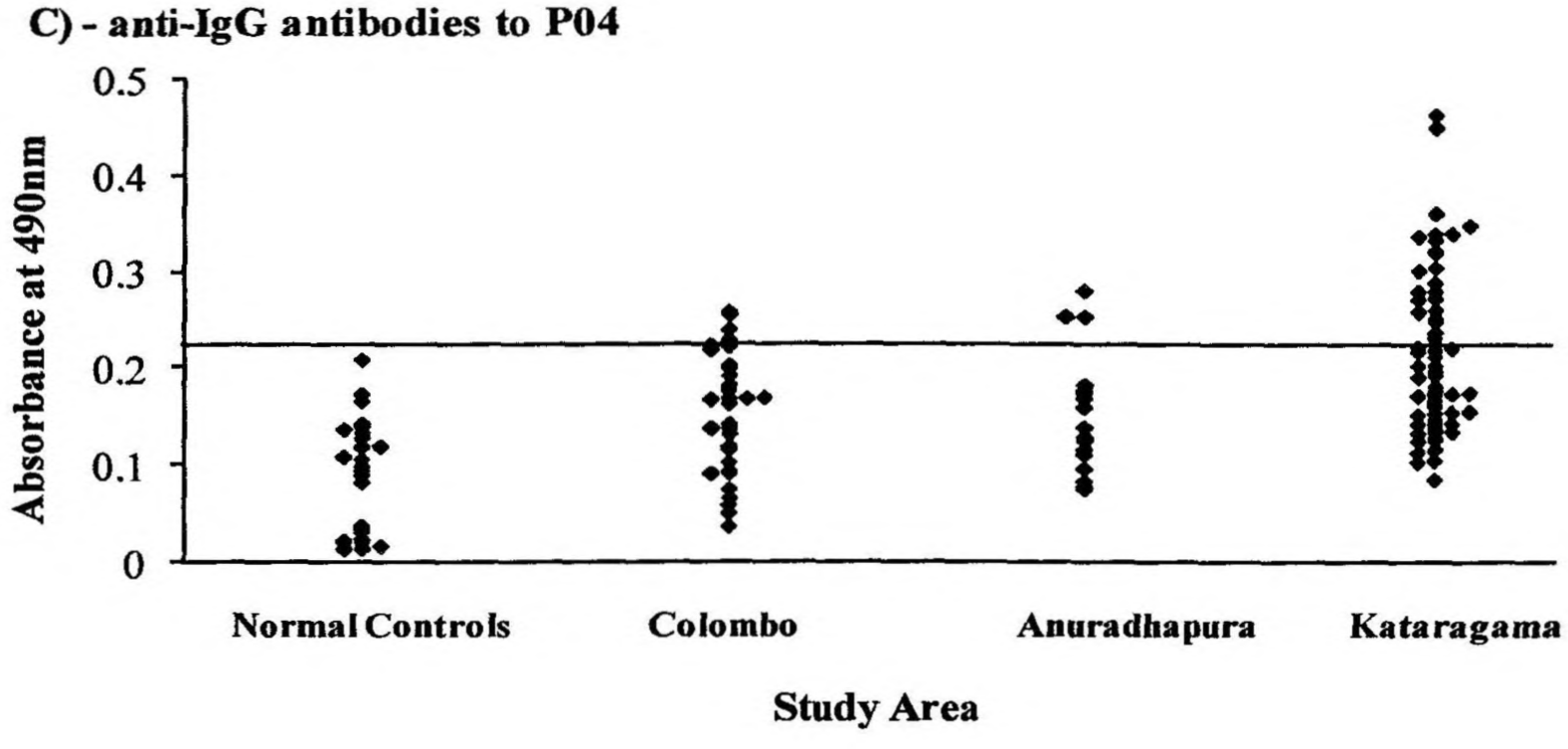
A significantly elevated IgM antibody magnitude was recorded from Anuradhapura compared to Colombo and Kataragama against P05 (Kruskal-Wallis test, $P<0.05$), while, though not significant a similar result was observed for IgM antibody magnitudes against P04. Further, a significantly elevated IgG antibody 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 5.6).

A) - anti-IgM antibodies to P04



B) - anti-IgM antibodies to P05





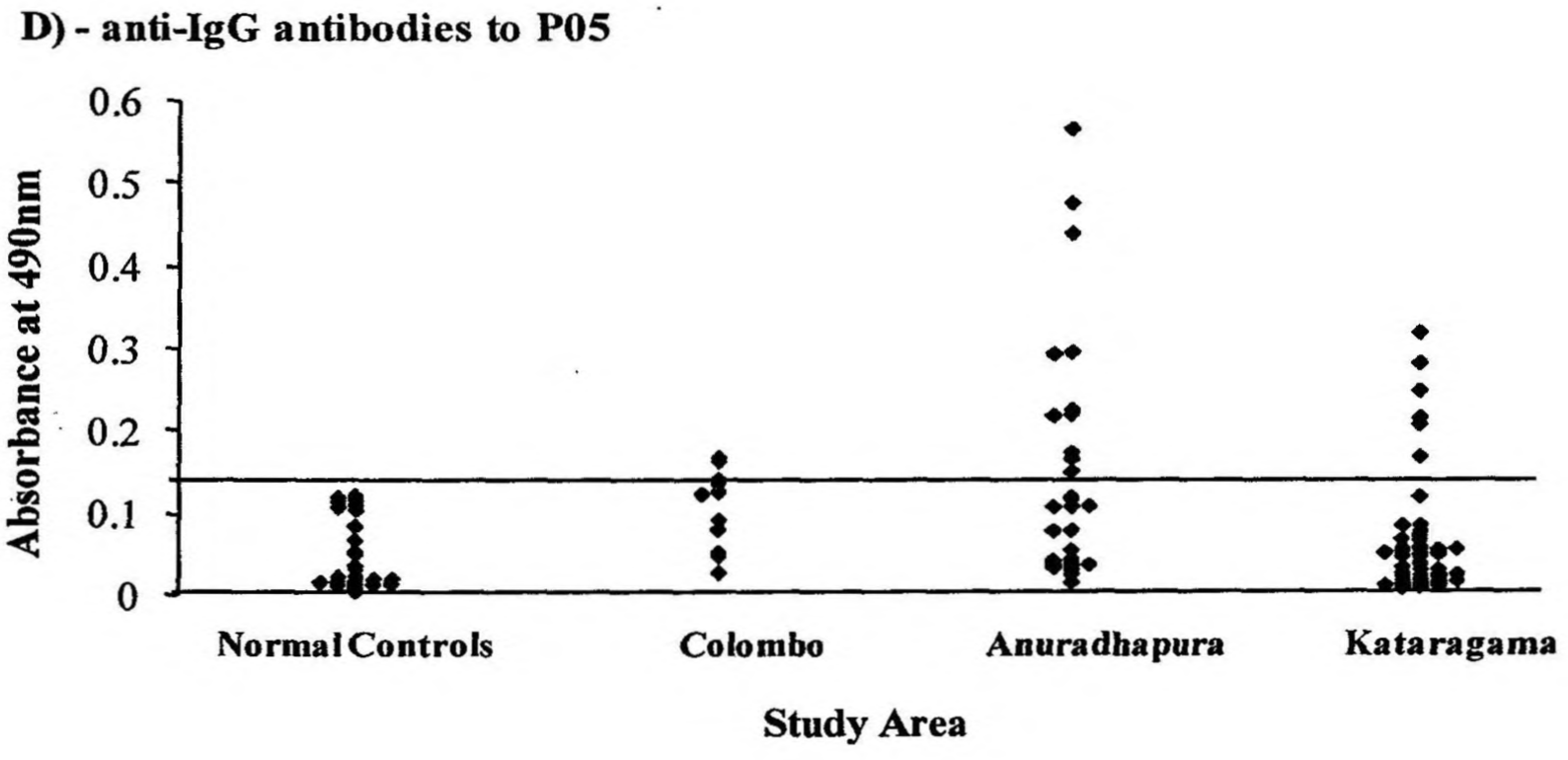


Figure 5.6. IgM and IgG subtype specific antibody responses of different study areas against anti-PvAMA-1 domain II P04 and P05 peptides

Each closed circle represents the mean value of duplicate absorbance values of each serum sample. The number of individuals in the normal control group and the test groups from Colombo, Anuradhapura and Kataragama were 28, 36, 33 and 65, respectively. The horizontal line indicates the cut off value (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.

Table 5.5. 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

Table 5.6. 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$_{490mn}$ 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

5.3.1.2.5. 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 5.7 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 5.7A). 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 5.7C). 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 resemblance of a subclass-specific shift towards cytophilic IgG antibodies was detected in P05 antibody response with increasing exposure in endemic residents ($P>0.05$), although this phenomenon was ambiguous than the P04 specific switch to cytophilic IgG.

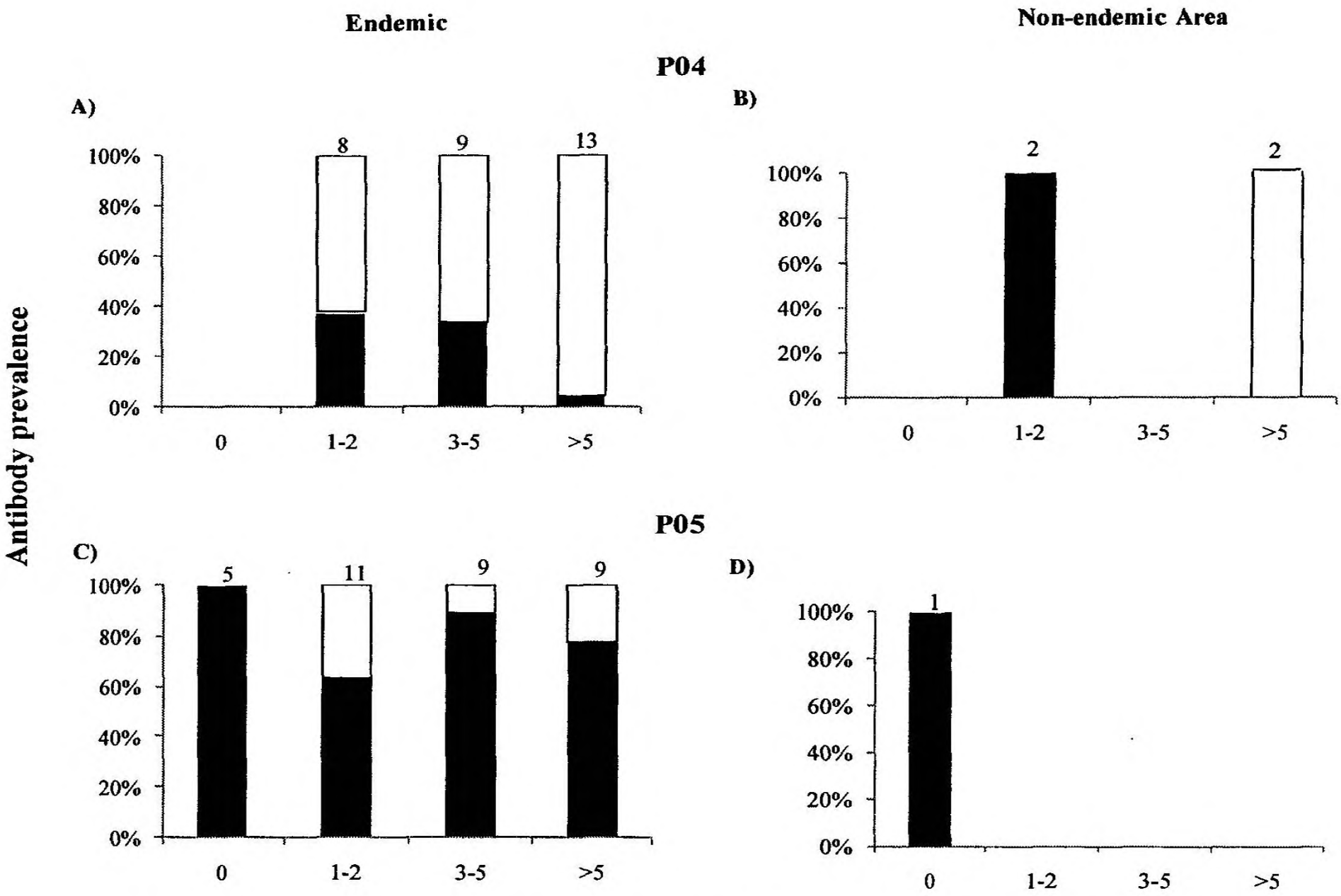


Figure 5.7. Pattern of anti-PvAMA-1 isotype-specific antibody prevalence with previous exposure to malaria

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

5.3.1.2.6. Preferential recognition of linear B cell epitopes against peptides P01-P07 of PvAMA-1 domain II

To determine the preferential antibody recognition of linear B cell epitopes of the seven synthetic peptides, six selected individuals were analysed from the three study areas for their antibody magnitudes against the seven peptides (Figure 5.8). Of the 162 samples analysed, only a single individual from Kataragama (LA05) recorded total anti-PvAMA-1 antibody magnitudes that exceeded control magnitudes. Interestingly, this individual has recorded a high positivity to peptides P04, P05 and P06 but very low positivity to P02, P03 and P07 in comparison to the respective control magnitudes. Conversely, CPV4467 from Colombo lacked peptide specific antibody magnitudes, as the magnitudes for all seven peptides were below those of the controls. The remaining individuals recorded preferential binding to one or more peptides (ASA 173, ASA 187, KTPV20, and CPV4482), as the antibody magnitudes corresponding to individual peptides fluctuated with that of the control magnitudes (Figure 5.8). According to these six selected individuals, those from Anuradhapura showed a high positivity to P05, while those from Kataragama had a high positivity to both P04 and P05. Strikingly, of the 162 individuals, only 9 from Colombo lacked peptide specific antibody magnitudes to all seven peptides. In conclusion, this analysis demonstrated the differential antibody recognition of linear B cell epitopes in different individuals against the seven synthetic peptides of PvAMA-1 domain II.

5.3.1.2.7. Correlation of the total antibody responses against the seven synthetic peptides with those observed for recombinant PvAMA-1

Attempts were made to establish correlation between the mean peptide-specific antibody magnitudes of all seven peptides of PvAMA-1 domain II and the antibody magnitudes against the recombinant PvAMA-1 protein (PV66/AMA-1) at individual level, of 142 serum samples from *P. vivax* infected individuals (Wickramarachchi, 2005). Figure 5.9 represents three clear groups of individuals who recognised i) the peptides but not the recombinant protein, ii) the recombinant protein but not the peptides and iii) both the recombinant protein and the peptides. Strikingly, a significantly high percentage of individuals recognised both the peptides and the recombinant PvAMA-1 protein (52%), compared to those with restricted

recognition of either the peptides (8%) or the recombinant protein (40%) (Chi square test = 67.33, P<0.001, Figure 5.9).

Moreover, a negative correlation was observed between the mean antibody magnitudes against the seven peptides and the recombinant PV66/AMA-1 protein, in individuals in group iii (r = -0.077, P>0.05). A significantly high percentage of individuals recognised both the rAMA-1 and the mean peptide-specific antibodies from Kataragama (43%) compared to Anuradhapura (38%) and Colombo (19%) (Chi square test = 10.86, P<0.05). When analysed separately among their study areas, though not significant, residents of Colombo (Figure 5.10A) and Anuradhapura (Figure 5.10B) demonstrated positive correlation between the antibody magnitudes against the rAMA-1 and the mean peptides, while those from Kataragama (Figure 5.10C) demonstrated a negative correlation (P>0.05). Though, a significant positive trend was observed between the prevalence of those individuals from Kataragama who recognised both the rAMA-1 and the peptides with increasing exposure (Chi square for linear trend = 18.85, P<0.001), the prevalence from those from Colombo depicted the contrary (P>0.05). A negative trend in the prevalence of individuals from Anuradhapura was observed from the exposure group 1-2 upwards (P>0.05).

Subsequently, associations were sort between the number previous malaria infections of vivax malaria patients in each study area with the antibody magnitudes against the rAMA-1 and the mean peptides separately. Although, individuals from Anuradhapura recorded a negative correlation (r = -0.426, P<0.05) to the antibody magnitudes against the rAMA-1 with increasing exposure, a positive correlation was recorded to the antibody magnitude against the mean peptides (P>0.05). Whereas, those individuals from Colombo and Kataragama recorded positive correlations between both the antibody magnitudes against the rAMA-1 and the mean peptides with increasing exposure (P>0.05). Strikingly, one individual from Kataragama who verbally indicated being exposed to the disease about 50 times, recorded a low antibody magnitude against the rAMA-1 and a high antibody magnitude against the mean peptides.

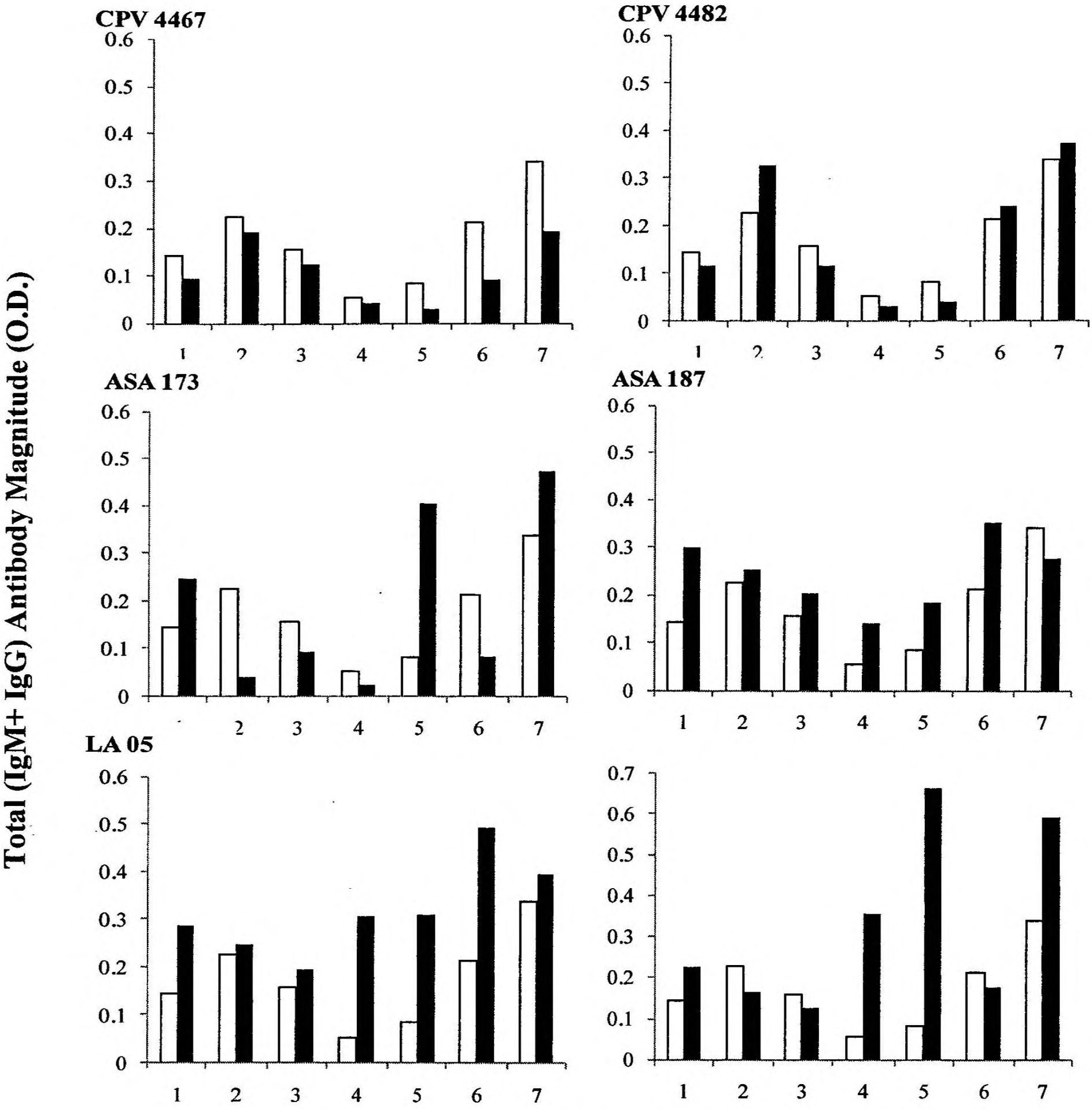


Figure 5.8. The antibody responses of a selected few *P. vivax* infected individuals from the three study areas against the synthetic peptides of PvAMA-1 domain II differentially recognised linear B cell epitopes

□ - represents the mean values + 2 SD for normal healthy controls (N=28) for each peptide.

■ - represents the antibody responses against all 7 peptides in six individuals.

CPV – Patient serum collected from Colombo

ASA – Patient serum collected from Anuradhapura

KTPV, LA – Patient serum collected from Kataragama

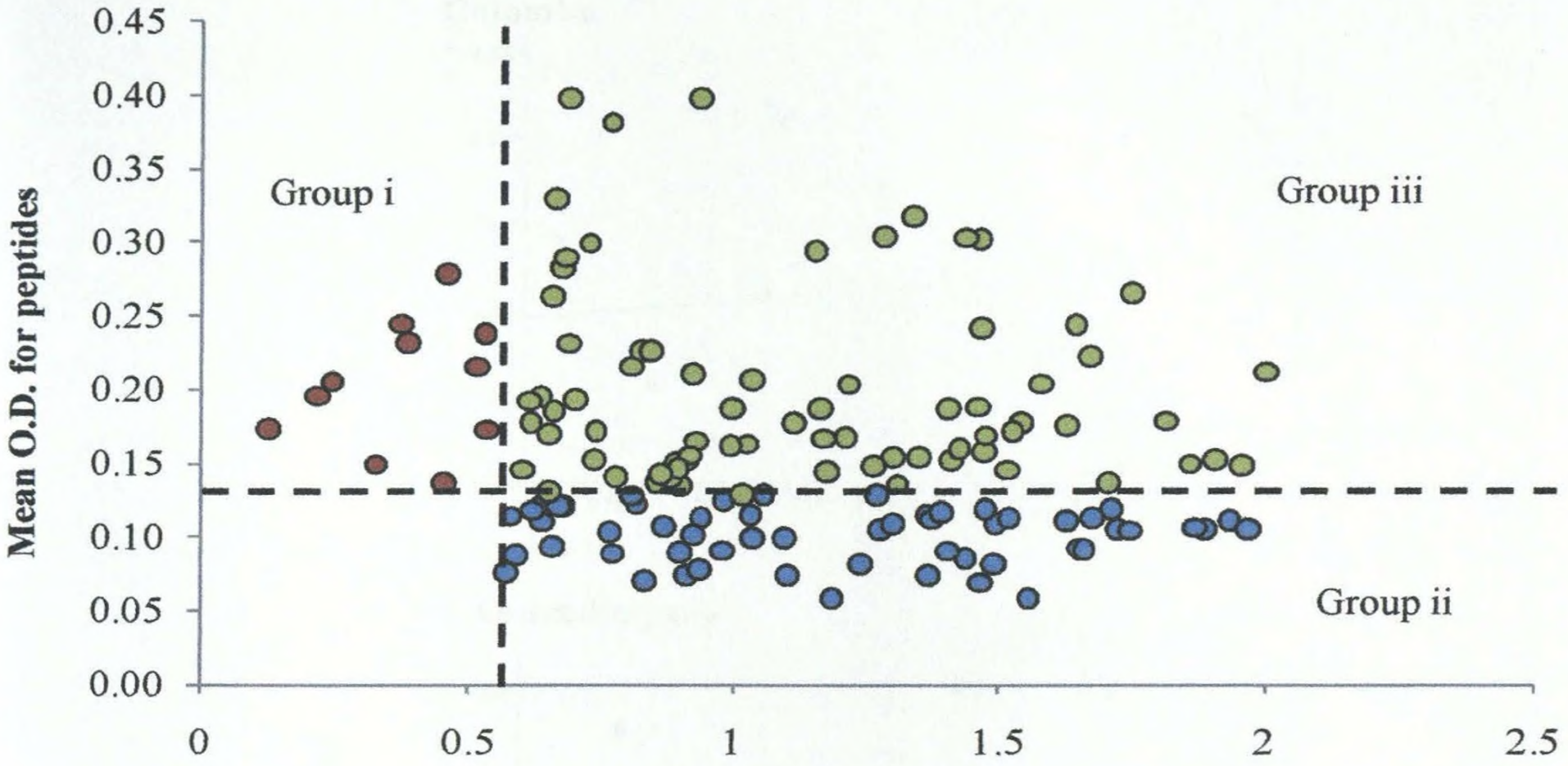


Figure 5.9. Correlation between the mean peptide specific antibody responses of PvAMA-1 domain II with that to rPvAMA-1, in *P. vivax* individuals from Sri Lanka

Peptide-specific antibody magnitudes were estimated by taking the mean OD of all 7 peptides. Each point represents serum of a single individual. The dashed line i) horizontally indicates the cut off for the mean peptide specific antibody response which is the mean+2SD for all peptides in 28 normal human controls and ii) vertically indicates the cut off for the PvAMA-1 recombinant protein (Pv66/AMA-1) previously assayed by Wickramarachchi, *et al.* (2007).

Individuals who recognised;

Group i - the peptides but not the recombinant protein

Group ii – the recombinant protein but not peptides

Group iii – both the recombinant protein and the peptides

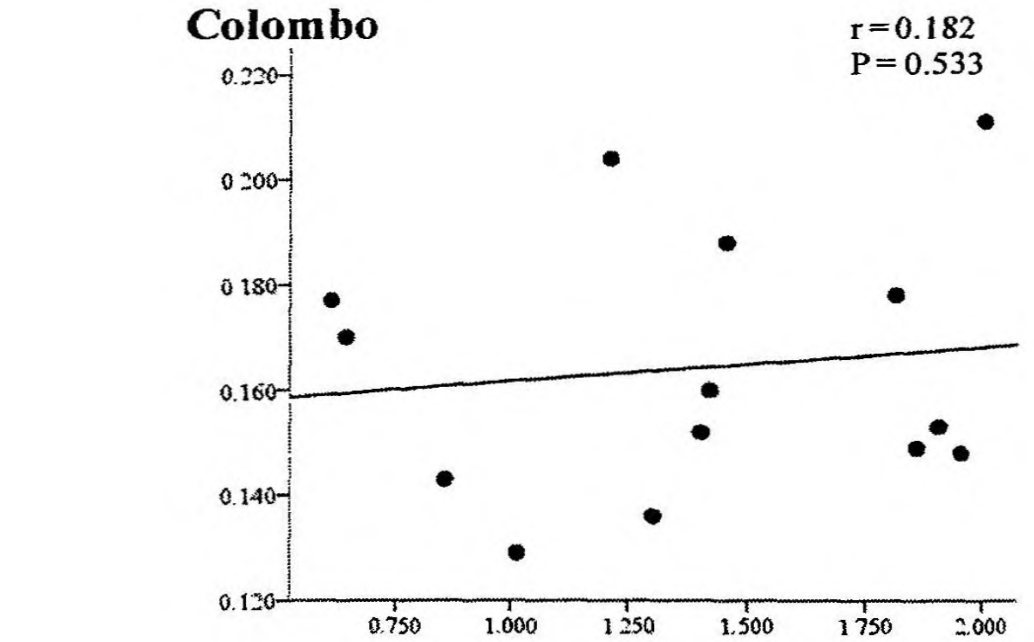


Figure 5.10. Correlation between antibody magnitudes of mean peptides and rPvAMA-1 with the number of previous malaria infections in individuals from the three study areas belonging to group III (those who responded both to the peptides and the recombinant protein)

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

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 5.11).

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. 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.

A similar analysis was carried out by comparing the anti-PvAMA-1 domain II antibody prevalence with that of the 12 amino acid polymorphic sites manifested in chapter 4. The first amino acid polymorphic residue in all seven peptides was identical to the PvAMA-1 domain II Sal I strain. An increase in anti-PvAMA-1 antibody prevalence was recorded against P02, P06 and P07 at amino acid positions 235, 310, 328, 338, 342, and 343 due to polymorphic substitutions occurring from E/K, K/N, F/L, Q/K, L/R and E/D, respectively. Conversely, a decline in anti-PvAMA-1 antibody prevalence at amino acid positions 211, 246, 310, 326, and 341 was observed against P01, P03, P06 and P07 due to polymorphic substitutions occurring from G/E, G/E, K/E, K/I, and D/V, respectively (Chi square test, $P>0.05$), while due to polymorphic substitutions at amino acid positions 329 and 338 from I/F and Q/R respectively, individuals lacked anti-PvAMA-1 antibodies against P07 (Figure 5.12).

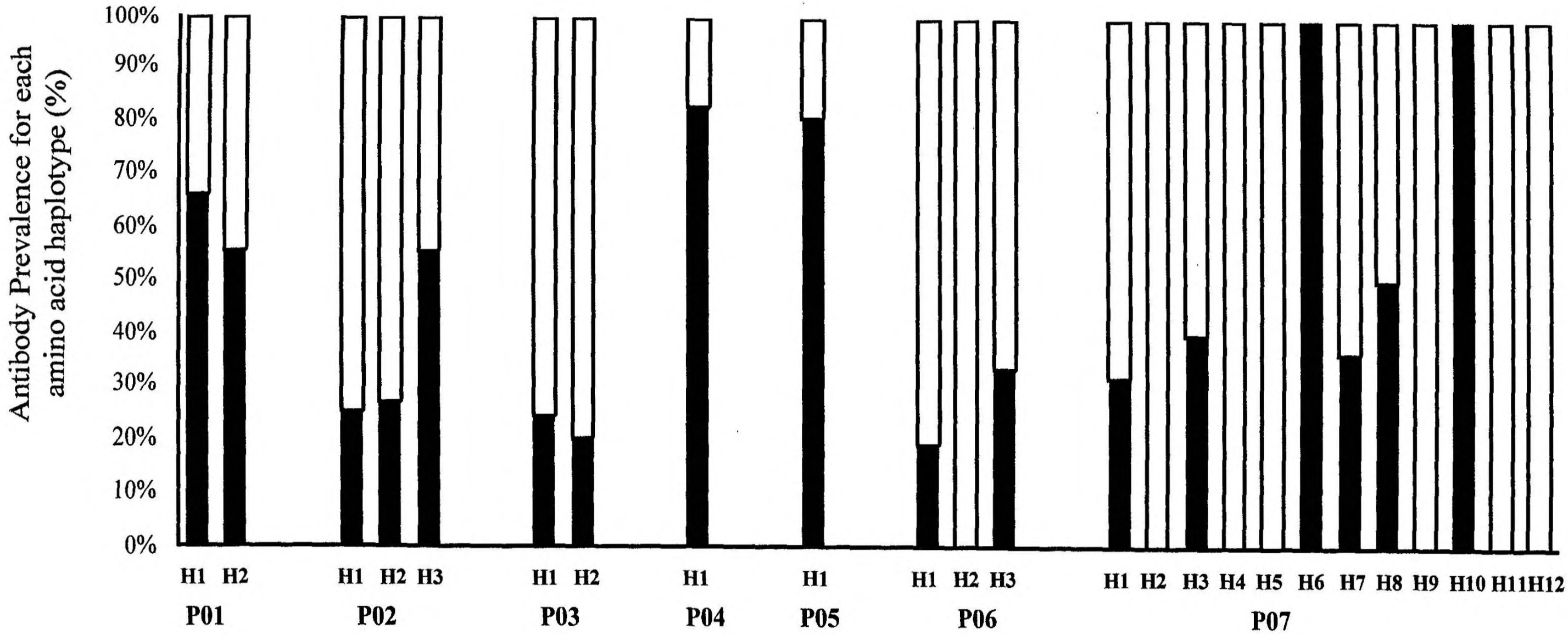


Figure 5.11. Distribution of the amino acid haplotypes to each of the seven synthetic peptides P01-P07 representing PvAMA-1 domain II, and the anti-PvAMA-1 domain II antibody prevalence against the seven synthetic peptides

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

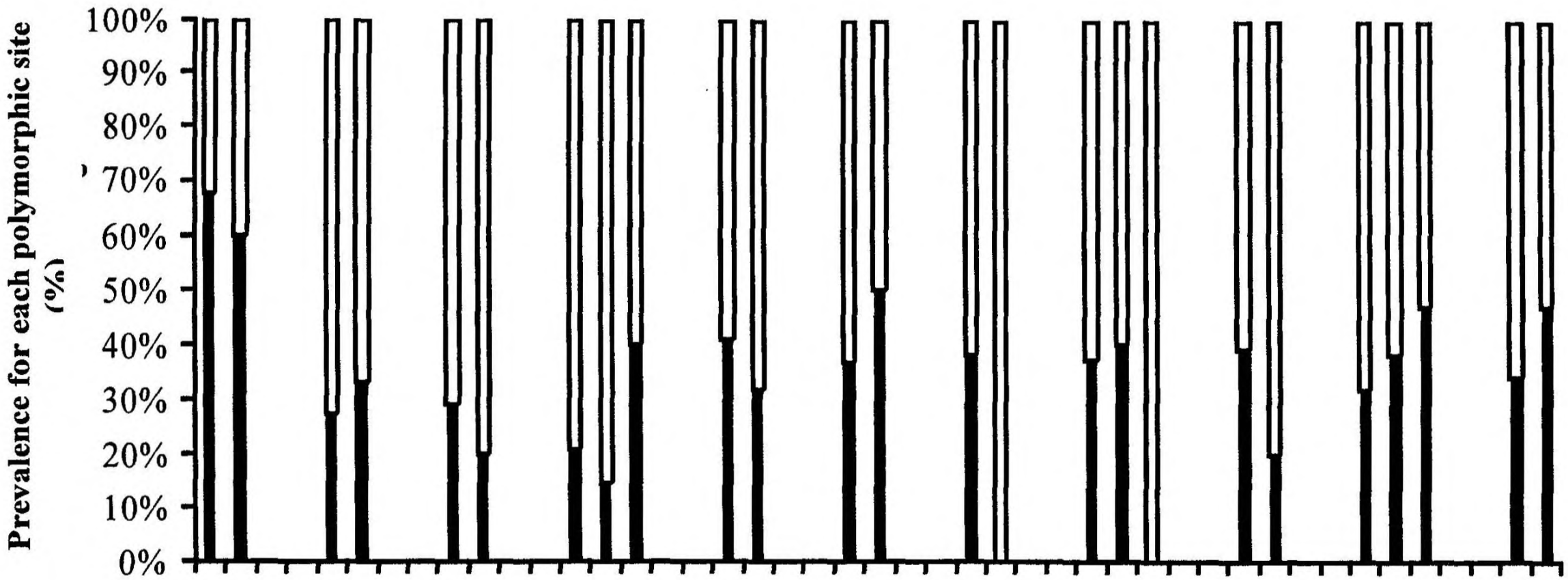


Figure 5.12. Distribution of the amino acid polymorphic positions to each of the seven synthetic peptides P01-P07 representing PvAMA-1 domain II, and the anti-PvAMA-1 domain II antibody prevalence against the seven synthetic peptides

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

5.3.3. Association between PvMSP-1_{42} amino acid haplotypes and total (IgM + IgG) antibody responses against the recombinant PvMSP-1_{42} and PvMSP-1_{19} proteins

Of the 95 amino acid sequences of PvMSP-1_{42} clinical isolates, 72 consisted antibody responses against the recombinant PvMSP-1_{42} representing the Belem strain and the PvMSP-1_{19}. 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 5.13). 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-1_{42} 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-1_{42} 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-1_{42} antibody prevalence, may imply strain-transcending (cross reactive) immune responses.

Further, anti-PvMSP-1_{42} antibody prevalence varied to haplotypes with identical homology percentage (but with dissimilar amino acid sequences) to the Belem PvMSP-1_{42}; 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-1_{42} 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-1_{42}, 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-1_{42} antibody prevalence against parasite haplotypes H6, H7, H9, H17 and H27 prove the immunogenicity of PvMSP-1_{42}.

Though, all 72 amino acid sequences of clinical isolates were identical to the PvMSP-1_{19} recombinant construct, the anti-PvMSP-1_{19} 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-1_{42} and PvMSP-1_{19}.

5.3.4. Combination of amino acid haplotypes from both PvMSP-1_{42} and PvAMA-1 domain II in Sri Lanka

Amino acid haplotype data on both PvMSP-1_{42} and PvAMA-1 domain II were available for a total of 39 *P. vivax* isolates from Sri Lanka. The combination of amino acid haplotypes from these two genes resulted in 33 different amino acid haplotype combinations termed as MA (Table 5.7). Of these, amino acid haplotype combination MA1, was predominatly distinctive to Kataragama (N=3, 8%), and constituted amino acid haplotypes H1 from the PvMSP-1_{42} and H1 from PvAMA-1 domain II. While, haplotype combinations MA4, MA8, MA21 and MA27 consisted two isolates each exclusively from Colombo, rest of the 28 amino acid haplotype combinations included single isolates each from Kataragama (43%), Colombo (39%) and Anuradhapura (18%). The predominant amino acid haplotype (H7) of PvMSP-1_{42} in Sri Lanka which was 92.9% homologous to the Belem 42 kDa fragment was combined with the amino acid haplotypes H13 and H19 of PvAMA-1 domain II, which were 97% and 98.5% homologous to the PvAMA-1 Sal I strain to form haplotype combinations MA6 and MA7.

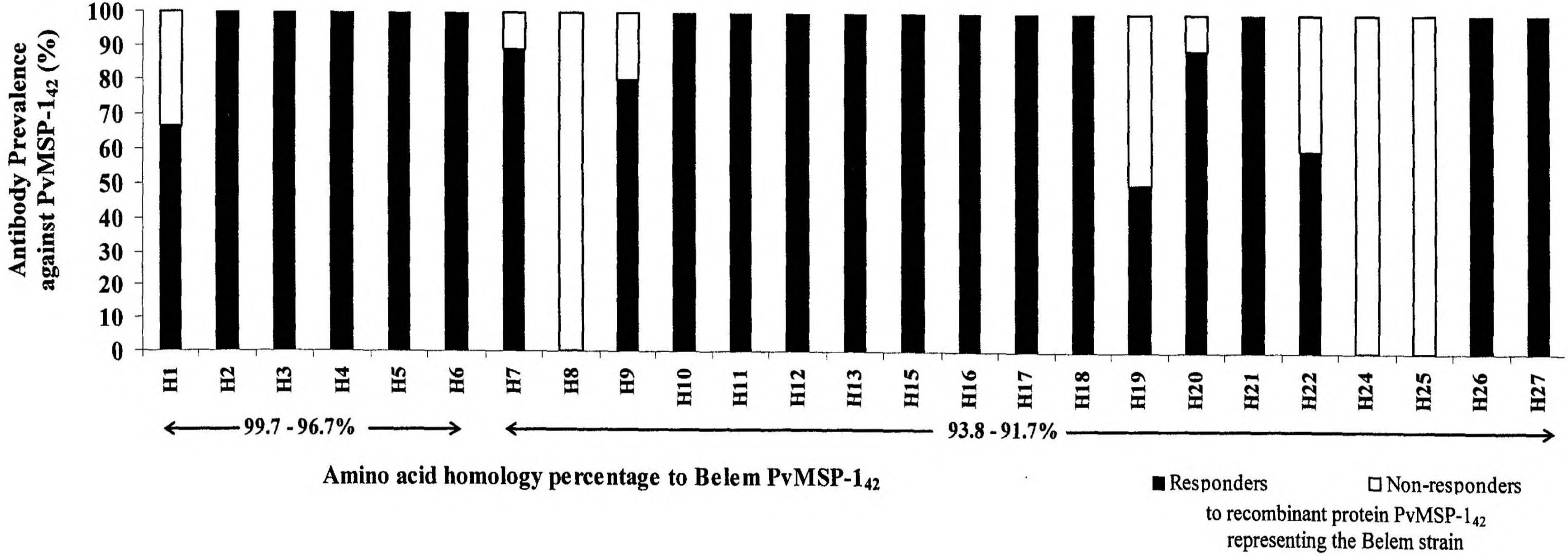


Figure 5.13. Distribution of the amino acid homology percentage to the PvMSP-1$_{42}$ Belem strain and the antibody prevalence against PvMSP-1$_{42}$ recombinant protein (Belem strain) of the manifested 27 amino acid haplotypes from Sri Lanka

Table 5.7. Amino acid haplotype combinations obtained from amino acid sequences of PvMSP-1_{42} and PvAMA-1 domain II in Sri Lankan *P. vivax* isolates

Amino Acid Haplotypes		Amino Acid Haplotype Combination[a]	Number of isolates representing each haplotype combination
PvMSP-1_{42}	PvAMA-1 domain II		
H1	H1	MA1	03
H2	H3	MA2	01
H2	H6	MA3	01
H4	H1	MA4	02
H5	H17	MA5	01
H7	H13	MA6	01
H7	H19	MA7	01
H9	H5	MA8	02
H9	H11	MA9	01
H11	H1	MA10	01
H12	H9	MA11	01
H13	H1	MA12	01
H13	H4	MA13	01
H13	H10	MA14	01
H13	H12	MA15	01
H14	H9	MA16	01
H15	H6	MA17	01
H16	H9	MA18	01
H17	H1	MA19	01
H17	H7	MA20	01
H17	H9	MA21	02
H19	H18	MA22	01
H20	H9	MA23	01
H20	H15	MA24	01
H22	H3	MA25	01
H22	H4	MA26	01
H22	H9	MA27	02
H22	H16	MA28	01
H23	H20	MA29	01
H25	H3	MA30	01
H27	H3	MA31	01
H27	H12	MA32	01
H27	H13	MA33	01

[a]: MA1 – MA33 represents the amino acid haplotype combinations obtained by MSP-1_{42} (M) and AMA-1DII (A) amino acid haplotypes

5.4. Discussion

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 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 contain a characteristic and genetically determined set of target antigens of protective immunity.

The seven peptides designed to represent domain II of the PvAMA-1 protein was based on a previous study carried out by Gunasekera *et al.* (2007) which demonstrated that in Sri Lanka a significant diversifying selection was acting on domain II. This was reiterated by a study, where bacterial recombinant proteins expressing each domain of PvAMA-1 or in combination (DI-DII or DII-DIII) demonstrated a high frequency of responders against recombinant proteins containing DII than those against DI or DIII (Múfalo *et al.* 2008). Moreover, ELISA inhibition assays using the ectodomain of PvAMA-1 as substrate, revealed the presence of many common epitopes within DI-II that were recognized by human immune antibodies. Therefore, domain II of PvAMA-1 was reinforced to be particularly immunogenic during natural human infections (Múfalo *et al.* 2008).

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 the 4G2 epitope of PfAMA-1. Additionally, this region overlapped with the amino acid sequence of peptide P04 of PvAMA-1 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.

Further, though, this study was based on the hypothesis that anti-PvAMA-1 antibody prevalence against each peptide is significantly higher in individuals living in malaria endemic areas than those in non-endemic Colombo, the total antibody prevalence against P04 among previously not exposed (PNE) patients was higher (73%) than for previously exposed (PE) patients (61%) from Colombo. This indicated that, though previous exposure to malaria could be a contributory factor for the presence of antibodies against PvAMA-1 (peptide P04 in this study), this peptide was immunogenic even in certain individuals with limited antigenic exposure.

Strikingly, as has been reported previously to the recombinant PvAMA-1 protein (Wickramarachchi *et al.* 2006), 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 in the present study. Thus, under low malaria 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 loosing 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 malaria endemic areas is IgG (Morais *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 to malaria, where majority of individuals were not exposed to the disease previously. 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 cystein residues that form disulfide bonds which are important for the folding of this molecule *in vivo*. Of the four cystein residues, two were located in the peptide sequence of P06, which is suggestive of the inclusion of conformational eptiopes. 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 antigens takes several years of exposure to parasites. This could explain the elevated positive trend observed in individuals from Kataragama to 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 low (N=9). Further, Mendis *et al.* (1990 & 1992) had 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 parasitemia 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.

Antibody directed to some linear B cell epitopes also recognize conformational (native) epitopes expressed on the merozoite surface and the recognition increases with cumulative exposure (Xainli *et al.* 2003). That majority of individuals recognised both the PvAMA-1 recombinant protein and the PvAMA-1 domain II peptides may indicate the recognition of both conformational and linear B cell epitopes. Antibody responses to these linear epitopes do not appear to develop with initial vivax malaria infection. This was reiterated by the positive trend observed in individuals from Anuradhapura between the previous exposures and the antibody magnitudes of PvAMA-1 domain II. Though, the positive trend evident between the total antibody prevalence against peptides P01, P02, P04, P05 and P07 and the number of previous malaria infection groups from Kataragama elaborates the above, a negative correlation was not observed between the antibody magnitudes against rAMA-1, except for one individual who claims to have been exposed to the disease 50 times or more. It was suggested that most residents of populations in areas endemic for *P. vivax*, however, recognize linear epitopes and the number of epitopes recognize increases with age and cumulative exposure (Xainli *et al.* 2003). However, in the current study the age and exposure ranged from 15-68 and 0-20, respectively which limited the observations made, compared to Xainli *et al.* (2003). Since a single *P. vivax* infection does not induce protective immunity, these initial antibody responses to immunodominant conformational epitopes may not confer resistance. Similarly to Xainli and colleagues (2003) interpretation of antibody responses to linear epitopes of DBP, repeated *P. vivax* infection may probably results in the release of

significant amounts of AMA-1, and along with its partial degradation it may stimulate antibody responses to linear epitopes. Partial protection may develop when sufficient antibody titers of high enough affinity are induced to multiple surface expressed determinants of AMA-1 that contain linear epitopes (Xainli *et al.* 2003). This is consistent with early observations of experimental *P. vivax* infection in humans that at least three to four infections of the same parasite strain were required to achieve protection against the disease and a significant reduction in parasitemia (Reviewed in Xainli *et al.* 2003). Though, Xainli *et al.* (2003) showed that residents of non-endemic areas of malaria who had become infected for the first time developed antibody responses only to the rDBP and not to linear epitopes, this observation was not reiterated for individuals from Colombo as some recognized both the recombinant PvAMA-1 and the PvAMA-1 domain II peptides .

Peptide P07 generated 7 amino acid substitutions, of which 4 corresponded to amino acid positions 326 (K/I), 338 (Q/K/R), 341(D/V) and 342(L/P/R) that included predicted linear B cell epitopes. A reduction of responding proportions were demonstrated in all these 4 sites with the deviation from the PvAMA-1 Sal I amino acid to another. Therefore, an immune response against a specific antigen may depend on the presence of dominant B cell epitopes and polymorphic residues can affect antibody binding, due to immune selection (Xainli *et al.*, 2003).

Among the 21 amino acid haplotypes identified at the PvAMA-1 domain II (chapter 4), 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. 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 (Cortés *et al.* 2005). 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, respectively lacked antibodies against these two peptides. 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). Of the 11 individuals who lacked total antibodies against all seven peptides, 9 responded against the recombinant PvAMA-1, PvMSP-1_{42} and PvMSP-1_{19} proteins which clearly indicates other contributory factors.

At PvMSP-1_{42}, those haplotypes that were more homologous to PvMSP-1_{42} Belem strain (H2-H6 with a 99.4-96.7% homology) demonstrated a strain-specific immune response against PvMSP-1_{42}. 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-1_{19} allelic forms in a *P. falciparum* infected natural human population of India. Presence of PfMSP-1_{19} allele specific antibodies observed in this population suggested that for a better protection against all strains of *P. falciparum*, it would be appropriate to use a vaccine containing all possible sequence variants (Mamillapalli *et al.* 2007).

However, a 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-1_{42} 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-1_{42} 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 amino acid haplotypes H8, H24 and H25 of PvMSP-1_{42} that lacked antibody responses against both 42, 19 kDa fragments of PvMSP-1 and PvAMA-1 (Wickramarachchi, 2005), together with those included in the 35.3% that did not respond to the recombinant PvMSP-1_{19} 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 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-1_{19} 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-1_{19}, manifested antibodies against the entire recombinant PvMSP-1_{42} protein, indicative of a probable antibody response solely against the 33 kDa region.

The present study demonstrated the widespread immunogenicity of the PvAMA-1 domain II derived synthetic peptides in malaria exposed individuals in Sri Lanka. Bharadwaj and colleagues (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 elicit 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 P07 peptide of PvAMA-1 domain II, peptide P04 clearly elicited an immune response amongst individuals in all three study areas, which consisted residues essential for the 4G2 conformational B cell epitope formation. Moreover, the protective immune response generated against PvAMA-1 domain II was apparent by the marked isotype switch from IgM to IgG against P04 with increasing exposure in residents from areas of endemicity. However, there are amino acids known to be crucial to the 4G2 epitope which is present on either sides of the P04 region (Remarque *et al.* 2007).

A strain-transcending immune response was clearly prevalent against the PvMSP-1_{42} protein, in that PvMSP-1_{42} strains differing in amino acid homology/sequence recognised the recombinant PvMSP-1_{42} protein resembling the native Belem construct. Though, anti-PvMSP-1_{19} antibody prevalence precludes strain-specific immune response against the 19 kDa frgment, this region clearly elicit a protective mature IgG immune response with increasing exposure (Wickramarachchi *et al.* 2007). The PvMSP-1_{42} conversely elicited an immature immune response, and the immunological "cross talk" between epitopes of PvMSP-1_{42} and PvMSP-1_{19} may interfere with the development of a mature IgG dominated protective antibody response, which in turn may complicate vaccine development (Wickramarachchi *et al.* 2007).

The results reported here signify that a construct representing the domain II loop region of PvAMA-1 and the 19 kDa region of PvMSP-1 may be effective in generating protective

immune responses against *P. vivax* infections in Sri Lanka. Mimotope approaches have resulted in the development of novel peptides that mimic the epitope of the invasion inhibitory antibody 4G2 (Casey *et al.* 2004). Subsequently, Sabo and co-workers (2007) designed a more active analogue of one of these peptides which showed a well defined region of structure by the nuclear magnetic resonance spectroscopy. They created a conformationally stabilized analogue by inserting a disulfide link with the aim of reducing flexibility, stabilizing the mean structure in solution and increasing the potency of the 4G2 mimotope. This analogue improved characteristics as an *in vitro* correlate of immunity, with the ability to detect antibodies to the 4G2 epitope in plasma of individuals exposed to *P. falciparum* malaria in PNG (Sabo *et al.* 2007). In conclusion, this approach could be taken into consideration for the generation of a protective vaccine construct against *P. vivax*.

CHAPTER 6

General Discussion

Malaria is responsible for much of human suffering and misery over the world, where during the last 10 years, the infection has been worsening in many areas of the world (World Malaria Report, 2008). Despite decades of intensive research, an effective vaccine against malaria is lacking. Efforts are being challenged by the complexity of the *Plasmodium* life cycle, its ability to parasitize and hide within the host cells, and its masterful ability to avoid clearance by the innate and adaptive host immune responses (Casares & Richie, 2009). Mainly, the extensive polymorphism observed in the surface antigens is commonly attributed to natural selection, which is assumed to favour polymorphism in those genes directly exposed to the vertebrate host's immune system as a strategic mechanism for evading the host's defence system (Escalante *et al.* 1998). Therefore, in order to make an effective vaccine, it will be perhaps necessary to incorporate design features capable of neutralizing the defence mechanisms used by the parasite, thereby enabling the host immune system to respond effectively. Neutralizing these defences may be equally important to drive immune responses against antigens to their maximum intensity (Casares & Richie, 2009).

Recent progress in Cell and Molecular Biology, Genetics, Immunology, Parasitology, Physiology and new mathematical models describing parasite–immune-cell interactions within a host has substantially improved our understanding of the infection and immune status of individuals. Together with newly developed molecular and diagnostic techniques, these advances help in quantifying infection and immunity at the population level (Hellriegel, 2001). Studies on pathogen genotypes and gene expression defined as molecular epidemiology, is just an extension of normal epidemiological analysis to incorporate any molecular information on pathogens detected within individuals. However, molecular information is often so rich that it requires specialized analytical schemes, in the form of population genetic parameters (Nash *et al.* 2005), phylogenetic trees (Joy *et al.* 2003; Mu *et al.* 2005), or gene expression profiles (Daily *et al.* 2005). Such schemes tend to determine the structure of the analysis that can be most usefully performed and generally yield more information than analyses that merely consider the presence or absence of particular genes or genotypes. The goal is to perceive beyond a description of variability to detect the effects of molecular and cellular processes in infection, pathogenesis, immunity, or therapeutic responses (Conway, 2007). Essentially, many heritable variations in human and mosquito populations exist alongside other environmental variations that affect the molecular

epidemiology of the parasite. Further, understanding the population structure of the malaria parasite, and how it is genetically distinct in different regions, may open up new avenues to area specific control measures (Newton, 2002).

Immunoepidemiology is an important part of the study of infectious diseases, as it combines immunology with epidemiology, which not only studies about the distribution and frequency of infection and disease but also uncover how types and levels on immunity varies overtime, in relation with exposure to infection or clinical presentation. At the individual level, this can reveal how factors such as frequency of infection or genetic variation affect levels of immunity and, at the community level, can reveal how immune responses influence the prevalence of infection and the burden of disease (Kwiatkowski, 2005). However, in many cases the observed patterns have proven to be difficult to interpret due to the underlying biological interactions and their ecological and genetic aspects and implications. These include the influences of the biological and social environment (Becker *et al.* 1995), and of the co-evolutionary history of parasites and their hosts (Ewald, 1994; Gupta & Hill, 1995). Furthermore, one should not be too surprised to find a lot of variability and heterogeneity in traits of the parasites (e.g. virulence, immunogenicity or transmissibility) as well as in those of the hosts (e.g. innate and acquired immunity or development of disease). Therefore, it is the responsibility of the immunoepidemiologists to take their theory to the field and carefully explain the implications of their results to seroepidemiologists, who in turn have to convince their colleagues in the lab to design experiments which are easier to relate to the situation in the field (Woolhouse, 1994).

Malaria epidemiology, as the result of the complex interplay among humans, vectors, and hosts, varies considerably in different geographic regions. Results from extensive research on *Plasmodium falciparum and P. vivax* population structures in the last decade have demonstrated that the parasite populations are highly heterogenetic in both hyperendemic regions and areas where malaria transmission is seasonal using both immunological as well as molecular biological techniques (Thomas *et al.* 1994; Bruce *et al.* 2000; Udhayakumar, *et al.* 2001; Cui *et al.* 2003; Ord *et al.* 2005; Rodrigues *et al.* 2005). In Sri Lanka, where malaria is unstable with low transmission conditions (Mendis *et al.* 1990), immuno and molecular epidemiological studies, have demonstrated the complexity of the disease (Udagama *et al.* 1990; Premawansa *et al.* 1993; Gunasekera *et al.* 2007; Wickramarachchi *et al.* 2006, 2007 & 2010).

According to Escalante and colleagues (1998), loci encoding proteins expressed on the surface of the sporozoite and the merozoite are more polymorphic than those expressed during the sexual stages or inside the parasite. These results agree with the general observation that stage-specific surface proteins exhibit high polymorphism when compared with internal antigens (Riley *et al.* 1994). *Plasmodium* merozoites are a major target of blood-stage malaria vaccine development because they are the only form that is exposed to the host immune system, particularly those proteins that are exposed to antibodies such as the ones on the surface, in the micronemes/rhoptries, and in the parasitophorous vacuole, including shed and soluble proteins. Inhibition of erythrocyte invasion by merozoites would in principle block the erythrocytic stage of the parasite life cycle and prevent clinical manifestations (Devi *et al.* 2007). The duration of a protein's accessibility to antibody is an important criterion and may explain why the parasite sheds all or most of its surface proteins at invasion. Considered as potential vaccine candidates, the Merozoite Surface Protein-1 (MSP-1) and the Apical Membrane Antigen-1 (AMA-1) displays high levels of heterozygosity and rates of evolution due to the outcome of the accumulation and frequent switch of suitable mutations, by means of which the parasite escapes the host's immune defences (Reviewed in Escalante *et al,* 1998).

An immunoepidemiological study carried out in Sri Lanka using the ectodomain of PvAMA-1 (PV66/AMA-1) and the 42 kDa fragment of PvMSP-1, demonstrated the immunogenic nature of both these antigens. However, the non-responsiveness to PV66/AMA-1 of approximately 45% of residents from areas of endemicity, and the immature antibody response against PvMSP-1_{42} polymorphic region may probably reflect genetically controlled mechanisms (Wickramarachchi *et al.* 2006 & 2007). Therefore, the current study was undertaken to understand the molecular epidemiology of these two antigens in Sri Lanka and to evaluate the possible effect of strain-specific antibody responses generated against the different amino acid haplotypes produced due to polymorphism of these genes.

The first comprehensive analysis of the diversity across the entire *Pvama-1* gene using a single population sample from Sri Lanka confirmed that, at the *ama-1* locus of *Plasmodium vivax* in Sri Lanka, the signature of diversifying selection was most strongly seen at domain II, indicating that the different domains in each species may be subject to varying selective pressures and functional constraints (Gunasekera *et al.* 2007). This was further corroborated by Thakur *et al.* (2008) in those clinical isolates from India while, Ord and colleagues (2008) observed a strong diversifying selection at domain I of the *ama-1* gene in both *P. vivax* and *P.*

falciparum infected isolates from Venezuela, where 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. Though, Venezuela is considered a hypoendemic area for malaria, alike Sri Lanka and India, where low malaria transmission persists (Mendis *et al.* 1990; Ord *et al.* 2008; Thakur *et al.* 2008), a majority of the manifested amino acid haplotypes from Venezuelan isolates were distributed within certain Sri Lankan and Indian haplotypes, while only a single amino acid haplotype was exclusively unique to the country (chapter 3). Therefore, it was evident that, clinical isolates from Venezuela which was part of the new world population that was introduced after European colonization have a somewhat reduced effective population size and thus reduced genetic diversity in comparison to old world populations (Grynberg *et al.* 2008). Hence, polymorphism at the *Pvama-1* domain II locus that is evidently subjected to immune-driven selection may be an important factor in the epidemiology of infection by *P. vivax*. Understanding the factors governing the extent and pattern of polymorphism at such loci may thus have implications for the development of effective control strategies (Hughes and Verra, 2002).

Consistent with results of *P. falciparum* (Chesne-Seck *et al.* 2005), lack of polymorphism was detected in a segment of the domain II loop of *Pvama-1* which included the epitope recognized by an anti–PfAMA-1 invasion-inhibitory mono clonal antibody (Pizarro *et al.* 2005). Functional constraints may limit antigenic variation in the loop, which is proposed to form a putative ligand-binding site within the PAN (*P*lasminogen, *A*pple, *N*ematode) domain, which defines a superfamily of protein folds implicated in receptor binding. Together, these data suggested that the constrained area defined by the domain II loop may serve as a potential vaccine component against *P. vivax* (Gunasekera *et al.* 2007). Though, the *ama-1* gene is considered to be highly conserved in *Plasmodium* species, this study demonstrated 82% of non-synonymous substitutions at *Pvama-1* domain II confirming the high allelic repertoire of this domain in parasites prevalent in the island where malaria transmission is low. Importantly, the similarity in observed single nucleotide polymorphisms (SNPs) between parasite isolates from Sri Lanka and isolates published elsewhere in India and Venezuela, signify a lack of gross regional diversity at *Pvama-1* domain II. It is imperative to evaluate the genetic diversity of *Pvama-1* gene in other geographic regions to reinforce this fact. However, a vaccine composed of *P. vivax ama-1* domain II may be equally effective in these malaria endemic populations though with marginal individual variation. Prior studies confirmed polymorphisms located on all three domains of the ecto-domain, suggesting that protective

epitopes are distributed over the entire antigen (Igonet *et al.* 2007). However, the distribution of these polymorphic sites was highly biased to one side of its three-dimensional structure (Gunasekera *et al.* 2007; Thakur *et al.* 2008) implying that a significant fraction of the surface contributed to by all three domains was subjected to functional constraints.

Single nucleotide polymorphisms (SNPs) have received considerable attention recently because of their potential as markers for genetic mapping and for studying molecular evolution and population dynamics (Reich *et al.* 2001; Sachidanandam *et al.* 2001). The single nucleotide polymorphisms at *Pvama-1* (in the current study at domain II, chapter 4) provide evidence for the suitability of *Pvama-1* domain II gene as a genetic marker in the evaluation of the genetic complexity of *P. vivax* infections locally and globally.

The observed anti-AMA-1 antibody prevalence to synthetic peptides P04 and P05 signified these two peptides to be highly immunogenic during *P. vivax* infections in Sri Lanka (chapter 5). As P04 and P05 peptides corresponded to the domain II loop of PvAMA-1 and were highly conserved to the Salvador I strain in all Sri Lankan test clinical isolates, the importance of this region in playing a prime role in protective immune mechanism is reinforced. However, the non-responding prevalence of 27% and 45% against P04 and P05 respectively, clearly over rule strain-specific immunity. Strikingly, the apparent isotype switch from a primary IgM response to a cytophilic IgG response with increasing exposure in the endemic study areas in Sri Lanka to the P04 peptide demonstrated the protective nature of the immune response of the domain II loop region represented by this peptide. Moreover, the amino acid sequence of the domain II loop of PvAMA-1 corresponded to amino acid fragments of P03 and P05 and to the entire length of P04, where amino acid residues of P04 corresponded to the amino acid region of the 4G2 epitope of PfAMA-1 domain II (Pizzaro *et al.* 2005). Moreover, according to Remarque *et al.* (2007), certain amino acid residues on either side of the epitope region was crucial to the 4G2 epitope, which fell on either side of the PvAMA-1 domain II P04 peptide. In conclusion, this study has provided ample evidence to design a vaccine inclusive of the domain II loop of PvAMA-1 in its required conformation to gain protection against *P. vivax* in individuals from Sri Lanka.

Extensive allelic diversity at the PvMSP-1_{33} locus was evident due to mutations, natural selection, and recombination. Strikingly, a majority of these mutations were clustered at the hypervariable region, where extensive interallelic and intragenic recombination gave rise to new MSP-1_{42} amino acid haplotypes in local and global *P. vivax* populations (chapter 4).

Interestingly, the phylogenetic tree evidenced a lack of geographic clustering of isolates, with neighbouring India and Sri Lanka observing common amino acid haplotypes due to frequent migration. Further, the population history of the old world and the new world were clearly visible from the phylogenetic tree where some Brazilian isolates were closely related to those isolates from Asia. On the contrary, PvMSP-1_{19} was highly conserved at the amino acid level in all Sri Lankan clinical isolates as well as in most of the global isolates. This is of significance as the reported crystal structure of PvMSP-1_{19} consists of a main binding pocket well suited for protein-protein interaction in the EGF domain I (Sarrano *et al.* 2006). This region is particularly important due to i) several growth inhibitory monoclonal antibodies (mAb) that bind to the first EGF domain of (Chappel & Holder, 1993) and ii) for the presence of a target epitope located on the N-terminal EGF-like domain of PfMSP-1_{19} (Pizzaro *et al.* 2003).

Although evidence indicates that the sequence divergence in PvMSP-1 was maintained over five million years by balanced selection exerted by the host's acquired immunity, the variant-specificity of naturally acquired antibodies to PvMSP-1 protein remains obscure (Putaporntip *et al.* 2006). In the current study, the naturally acquired antibodies against different PvMSP-1_{42} strains in the local population recognised recombinant PvMSP-1_{42} resembling the native Belem construct confirming the presence of cross reactive epitopes associated with strain-transcending immunity to *P. vivax* in Sri Lanka (chapter 5). Tertiary structure predicted for the hypervariable region of PvMSP-1_{42} suggests that this domain may form a highly exposed immunogenic loop comprising of many cross reactive B-cell epitopes (Manamperi, 2002). Though, PvMSP-1_{19} precluded a strain-specific immune response, this region clearly elicited a protective mature IgG antibody response with increasing exposure in those individuals from the endemic areas. Comparison of naturally acquired antibody responses to the C-terminal processing products of PvMSP-1 carried out by Wickramarachchi *et al.*(2007) confirmed that the antibody prevalence to the two proteins at the individual and population levels, tend to respond more to PvMSP-1_{42} than to PvMSP-1_{19} in all study areas. Further, with increasing exposure to malaria in both endemic areas, anti-PvMSP-1_{19} antibody responses were dominated by the functionally important IgG1 and IgG3 isotypes, with a concurrent reduction in IgM. In contrast to PvMSP-1_{19}, PvMSP-1_{42} antibodies principally reflected a primary IgM response irrespective of the patient's previous exposure. These data suggest that a cross reactive PvMSP-1_{42} ''immature'' antibody response, with a high proportion of IgM antibodies directed against the p42 polymorphic region, may interfere with a PvMSP-1_{19} ''protective'' isotype shift. Sachdeva *et al.* (2004) carried out an immunodepletion assay to characterize the

relative contributions of PvMSP-1_{42} and PvMSP-1_{19} specific epitopes in the human anti-PvMSP-1 response, by comparing the reactivity of the depleted sera with PvMSP-1_{42} antigen with that of the undepleted sera, by absorbing antibodies to PvMSP-1_{19} antigen in pooled patient sera in such a way that all of the PvMSP-1_{19} specific antibodies were lost. It implied that, certain PvMSP-1_{19}-specific epitopes remain cryptic in the structure of PvMSP-1_{42} and are exposed to the immune system only after secondary processing has taken place in PvMSP-1_{42} to yield PvMSP-1_{19}. Similar results have been reported for baculovirus expressed *P. vivax* and *P. cynomolgi* antigens based on the corresponding MSP-1 (Holm, *et al.* 1997). Overall, this reiterates the suggestion that immunological ''cross-talk'' between epitopes of PvMSP-1_{42} and PvMSP-1_{19} may interfere with the development of a mature IgG dominated protective antibody response, which may complicate vaccine development proposed by Wickramarachchi *et al.* (2007). PvMSP-1_{19} may be a better vaccine candidate antigen, since majority of the geographically different isolates were found to be highly conserved at the PvMSP-1_{19} region and there seems to be no compromise in the immune response, both qualitatively and quantitatively, when it is used alone. However, 35.3% were non-responsive to this protein in Sri Lanka and majority of these patients were from endemic Kataragama (Wickramarachchi *et al.* 2007), which is a typical *P. vivax* endemic area in Sri Lanka with a high annual parasitic incidence (API) (Gunawardena *et al.* 1994; Briët *et al.* 2003). It has been shown that, immunosupression/ immunodepression can be logical factors governing the effects of non-response (Pachner, 1997). Therefore, a vaccine generated against PvMSP-1_{19} alone might pose a problem especially in geographically different areas where malaria transmission is high. It will be useful to assess the protective efficacies of this antigen in combination with other vaccine candidate antigens.

It has been proposed, that a vaccine against a single antigen is unlikely to confer complete protection. A multi-stage, multi-component "cocktail" malaria vaccine containing different antigens would be the final goal of vaccine development to combat this major disease (Pan *et al.* 2004). Technologies being used include recombinant proteins, synthetic peptides and DNA vaccines, where the relevant genes encoding malaria antigens are inserted into a plasmid or into a live vector such as vaccinia or poxvirus. A number of human trials using different antigens and technologies have been carried out recently in the search for a malaria vaccine that can reduce the burden of disease in the most affected populations. Each trial provided additional knowledge about mechanisms of protection as well as about the effectiveness of new vaccine technology (Holder, 2009). However, the efficacy of a vaccine is hindered by the antigenic polymorphism elicited by the parasite to escape the host immune system. In the

current study, the 33 amino acid haplotype combinations manifested from PvMSP-1_{42} and PvAMA-1 domain II suggested a complex genetic diversity present in *P. vivax* in Sri Lanka, which will hinder vaccine development. Next, the query arises whether the sequence differences among each antigen is indicative of functional antigenic polymorphism at the level of antibody responses ensuing strain-specific immunity. Hence, it is crucial to identify multiple protective domains or epitopes from different antigens that are protective against the natural infection. Thus, it would be fruitful to focus on target antigens which may be relatively less immunogenic, but which are conserved among natural isolates (Manamperi, 2002). In this regard, the current study demonstrated the significance of the highly conserved domain II loop of PvAMA-1 and PvMSP-1_{19} as both the P04 peptide of PvAMA-1 domain II and the PvMSP-1_{19} fragment elicited a protective antibody response by the isotype switch from primary IgM to functionally important cytophilic IgG with increasing exposure to malaria in areas of endemicity.

It is of interest to define B and T cell epitopes of major malaria vaccine target antigens for their inclusion in subunit vaccine constructs (Joshi *et al.* 2000). The current study analyzed the humoral immune responses to these peptide epitopes in malaria exposed human subjects from areas of endemicity in Sri Lanka. Though it was also critical to study the cellular immune responses by way of including lymphocyte proliferation assays followed by cytokine analysis, the current study could not accommodate this analysis due to logistical drawbacks such as time and funding limitations. If an activation of mixed Th1+Th2 immune responses is shown protection will be verified. Lalitha *et al.* (2008) investigated the cellular immune responses to the recombinant AMA-1 Domain I+II formulation in immunized mice using a lymphocyte proliferation assay and a cytokine assay which resulted in significant proliferative responses and cytokines. Further those antibodies activated by immunization were biologically active in *P. falciparum* parasites as seen from the growth inhibition/invasion assay. However, a similar study for *P. vivax* is unfeasible due to the restriction of long term culture of the asexual blood and gametocyte stages of *P. vivax* (Galinski and Barnwell, 2008).

Polyvalent subunit malaria vaccines in contrast to single antigen based vaccines would be more potent in inducing "multiple layers" of immunity (Devi *et al.* 2007). In this regard, combination of merozoite antigens such as MSP-1_{19}, AMA-1 and DBP will concentrate the point of antibody attack at the machinery of invasion by simultaneously neutralizing multiple ligands with different functions critical to the parasites survival. This study supports the potency of using correctly reproduced complex conformation B cell epitopes and protective T

cell epitopes of the PvMSP-1_{19} and the domain II loop of PvAMA-1 as veritable vaccine components in a multi component recombinant protein to develop "protective" immune efficacy to target the asexual stages of *P. vivax* under local geographic settings. Such efforts would be further enhanced by the addition of the critical binding region of the PvDBPII.

With this perspective, a recombinant protein consisting of the PvMSP-1_{19}, the domain II loop of PvAMA-1 and the critical binding region of PvDBPII may be used to immunize adult toque monkeys (*Macaca. sinica sinica*) in Sri Lanka to study the immunogenicity and protective efficacy.

Nevertheless, as current vaccine development programmes are focussed on sustainable disease control, a partially effective asexual stage vaccine may not be useful in the elimination of parasites. However, malaria elimination which is the new goal endorsed by the World Health Organization, is proposed to be achieved by targeting pre-erythrocytic, asexual and sexual stages of the parasite, with multi-component, multi-stage vaccine(s) effecting mainly on both the disease and the infection. An expanded malaria vaccine programme with such objectives is now a priority (Targett & Greenwood, 2008).

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Research communications and publications accrued from the research project

Research communications:

a) Presented at International Conferences

1. 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.

2. Sajani Dias, Thilan Wickramarachchi, Shirley Longacre and Preethi V. Udagama-Randeniya (2010). Strain specific immune responses against *Plasmodium vivax* Merozoite Surface Protein-1p42 (PvMSP-1p42) and p19 (PvMSP-1p19) strains in Sri Lanka. 14th International Congress of Immunology, Kobe, Japan, 22-27, Aug, 2010.

b) Presented at National Conferences

1. 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, December 2006. Page3.

2. 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, September 2008. Page 42.

3. 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.

4. 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.

5. 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.

6. 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.

7. Sajani Dias, Shirley Longacre and Preethi V. Udagama-Randeniya (2010). Intrallelic and Intragenic Recombination at the Hypervariable Region of Merozoite Surface Protein-1 (MSP-1_{33}) of *Plasmodium vivax* in Sri Lanka. Post-graduate Research Symposium, Faculty of Science, University of Colombo. Page 111-112.

Publications

1. 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 PvMSP3α gene, in Sri Lanka. Annals of Tropical Medicine and Parasitology, 104(2); 95-108. In Press.
   (* Equal contribution to this work)

Manuscripts in preparation

1. 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 in preparation to be submitted to the journal of Infection, Genetics and Evolution.

2. 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 Molecular Immunology.

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 µl
0.5M EDTA	-	100 µl
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 - 50X TAE buffer

Tris-HCL	242 g
Glacial acetic acid	57.1 ml
0.5M EDTA, pH 8.0	100 ml
Distilled water up to	1000 ml

Appendix IV - Optimization of different antigen concentrations for the analysis of total (IgM + IgG) and sub class specific antibodies

IgM + IgG:

Peptide	Optimum
P01	4.5 µg/ml
P02	3.5 µg/ml
P03	8 µg/ml
P04	10 µg/ml
P05	3 µg/ml
P06	4 µg/ml
P07	6 µg/ml

IgM:

Peptide	Optimum
P04	14 µg/ml
P05	15 µg/ml

IgG:

Peptide	Optimum
P04	14 µg/ml
P05	11 µg/ml

Appendix V - Optimization of anti-human mouse monoclonal concentrations for the analysis of sub class specific antibodies

Monoclonal	Antigen	Optimum dilution
IgM	P04	1:1500
	P05	1:3500
IgG	P04	1:750
	P05	1:750

Appendix VI –

Optimization of Horseradish peroxidase linked anti-human Rabbit monoclonal concentrations for the analysis of total antibodies

Monoclonal	Antigen	Optimum dilution
IgM + IgG	P01	1:1250
	P02	1:1500
	P03	1:2250
	P04	1:2500
	P05	1:2000
	P06	1:2000
	P07	1:750

Optimization of Horseradish peroxidase linked anti-mouse Goat monoclonal concentrations for the analysis of sub class specific antibodies

Monoclonal	Antigen	Optimum dilution
IgM	P04	1:1500
	P05	1:3500
IgG	P04	1:750
	P05	1:750

Appendix VII – 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_2PO4 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 VIII– Blocking Buffer

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

Appendix IX– Washing buffer

0.2% Tween 20 (Polyoxyethylenesorbitan monolaurate, Sigma, USA) in 1X PBS (pH=7.4) for the analysis of total antibodies.

1% Tween 20 (Polyoxyethylenesorbitan monolaurate, Sigma, USA) in 1X PBS (pH=7.4) for the analysis of sub class specific antibodies.

Appendix X – Dilution Buffer

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

Appendix XI – Substrate Buffer

Citric acid - 5.92 g
Na_2HPO_4 (anhydrous) - 6.19 g

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

Appendix XII – Sulphuric Acid (2N)

Conc. H_2SO_4 - 15 ml

Mixed with distilled water and made the final volume up to 1000ml.

Appendix XIII – Data Table

Area	Serial number	Total response (IgG+IgM)							IgG		IgM			Age	NPI[1]	P[2]
		P01	P02	P03	P04	P05	P06	P07	P04	P05	P04	P05	IgM			
1	CPV4250	0.078	0.080	0.062	0.194	0.029	0.222	0.161	0.166	.	0.041	.		26	0	.300
1	CPV4261	0.099	0.109	0.072	0.133	0.029	0.237	0.220	0.201	.	0.057	.		32	4	.
1	CPV4275	0.049	.	0.064	0.143	0.024	0.132	0.178	0.167	.	0.063	.		23	2	.600
1	CPV4298	0.111	0.102	0.086	0.202	0.048	0.153	0.297	0.238	.	0.133	.		22	1	.
1	CPV4338	0.102	0.076	0.050	0.127	0.048	0.067	0.294	0.220	.	0.086	.		39	0	.200
1	CPV4348	0.038	0.069	0.042	0.293	0.024	0.068	0.200	0.161	.	0.135	.		33	1	.
1	CPV4356	0.068	0.070	0.034	0.153	0.031	0.034	0.253	0.137	.	0.054	.		24	1	.180
1	CPV4368	0.087	0.102	0.059	0.196	0.021	0.084	0.253	0.174	.	0.022	.		22	3	.030
1	CPV4383	0.043	0.101	0.038	0.062	0.010	0.056	0.201	0.090	.	0.039	.		33	5	.010
1	CPV4384	0.051	0.093	0.050	0.089	0.022	0.056	0.248	0.181	.	0.072	.		38	2	.150
1	CPV4385	0.053	0.081	0.039	0.116	0.029	0.053	0.161	0.177	.	0.066	.		49	0	.050
1	CPV4407	0.026	0.057	0.024	0.074	0.031	0.054	0.138	0.136	.	0.050	.		43	1	.250
1	CPV4416	0.048	0.079	0.051	0.224	0.040	0.052	0.232	0.135	.	0.116	.		23	0	.020
1	CPV4418	0.026	0.065	0.024	0.066	0.025	0.262	0.148	0.074	.	0.059	.		27	14	.100
1	CPV4423	0.038	0.072	0.054	0.113	0.024	0.067	0.202	0.130	.	0.027	.		28	9	.100
1	CPV4425	0.019	0.070	0.027	0.046	0.021	0.045	0.255	.	.	.	.		27	1	.200
1	CPV4439	0.050	0.261	0.129	0.116	0.043	0.117	0.351	0.167	.	0.041	.		25	0	.050
1	CPV4440	0.030	0.111	0.098	0.059	0.033	0.077	0.315	0.118	.	0.045	.		45	0	.010

1	CPV4447	0.026	0.095	0.092	0.043	0.018	0.057	0.213	.	.	.	.	.	1	.060
1	CPV4452	0.036	0.069	0.100	0.139	0.085	0.156	0.012	.	.	.	.	29	10	.005
1	CPV4453	0.153	0.286	0.191	0.152	0.070	0.048	0.526	0.190	.	0.088	.	37	0	.
1	CPV4461	0.142	0.384	0.150	0.032	0.018	0.113	0.400	.	.	.	.	24	0	.001
1	CPV4462	0.117	0.336	0.133	0.041	0.045	0.026	0.421	.	.	.	.	33	2	.
1	CPV4467	0.094	0.195	0.124	0.043	0.029	0.093	0.195	.	.	.	.	18	2	.200
1	CPV4468	0.188	0.176	0.083	0.030	0.042	0.066	0.243	.	.	.	.	48	1	.350
1	CPV4471	0.163	0.202	0.111	0.046	0.085	0.061	0.221	.	0.028	.	0.085	28	0	.
1	CPV4472	0.168	0.238	0.145	0.259	0.237	0.071	0.320	0.198	0.134	0.083	0.006	28	0	.
1	CPV4473	0.170	0.174	0.124	0.044	0.041	0.059	0.176	.	.	.	.	22	10	.
1	CPV4474	0.234	0.282	0.204	0.065	0.089	0.097	0.345	0.256	0.079	0.078	0.026	.	15	.070
1	CPV4476	0.138	0.186	0.112	0.038	0.060	0.051	0.152	.	.	.	.	15	0	.
1	CPV4480	0.087	0.218	0.132	0.076	0.034	0.063	0.342	0.141	.	0.046	.	22	2	.
1	CPV4481	0.152	0.276	0.155	0.043	0.072	0.066	0.425	.	.	.	.	22	3	.
1	CPV4482	0.117	0.327	0.118	0.033	0.041	0.241	0.372	.	.	.	.	33	4	.
1	CPV4491	0.225	0.306	0.113	0.189	0.056	0.166	0.425	0.217	.	0.025	.	49	0	.100
1	CPV4493	0.146	0.175	0.060	0.148	0.160	0.126	0.231	0.115	0.052	0.077	0.020	29	0	.300
1	CPV4495	0.132	0.133	0.041	0.052	0.024	0.099	0.151	.	.	.	.	23	1	.
1	CPV4501	0.065	0.240	0.059	0.031	0.020	0.103	0.211	.	.	.	.	.	5	.
1	CPV4507	0.069	0.161	0.081	0.308	0.022	0.253	0.151	0.223	.	0.018	.	26	2	.150
1	CPV4508	0.070	0.162	0.067	0.035	0.034	0.103	0.163	.	.	.	.	38	1	.080
1	CPV4524	0.078	0.173	0.101	0.092	0.030	0.177	0.166	0.101	.	0.016	.	22	1	.025
1	CPV4526	0.130	0.308	0.144	0.035	0.024	0.085	0.344	.	.	.	.	26	.	.125
1	CPV4534	0.047	0.205	0.117	0.023	0.024	0.100	0.222	.	.	.	.	30	4	.030

1	CPV4537	0.068	0.099	0.098	0.019	0.014	0.140	0.130	.	.	.	.	23	2	.020
1	CPV4540	0.096	0.213	0.089	0.046	0.041	0.160	0.257	.	.	.	.	45	0	.010
1	CPV4557	0.140	0.137	0.124	0.064	0.016	0.062	0.230	0.058	.	0.015	.	37	4	.270
1	CPV4566	0.236	0.215	0.178	0.029	0.044	0.115	0.221	.	.	.	.	.	.	.
1	CPV4575	0.163	0.124	0.146	0.020	0.017	0.140	0.180	.	.	.	.	21	2	.
1	CPV4576	0.182	0.139	0.172	0.247	0.165	0.137	0.214	0.253	0.140	0.049	0.014	.	.	.
1	CPV4727	0.224	0.158	0.164	0.157	0.163	0.162	0.329	0.222	0.122	0.022	0.013	23	0	.010
1	CPV4731	0.130	0.133	0.147	0.401	0.150	0.117	0.199	0.227	0.081	0.024	0.022	29	2	.060
1	CPV4733	0.075	0.128	0.148	0.099	0.088	0.152	0.204	0.093	0.048	0.085	0.046	54	1	.090
1	CPV4735	0.095	0.092	0.140	0.079	0.117	0.048	0.094	0.065	0.091	0.027	0.022	31	1	.130
1	CPV4741	0.218	0.070	0.112	0.141	0.168	0.142	0.351	0.090	0.124	0.029	0.034	19	1	.010
1	CPV4743	0.078	0.061	0.071	0.203	0.148	0.264	0.151	0.049	0.161	0.036	0.039	30	.	.080
1	CPV4750	0.205	0.166	0.453	0.096	0.141	0.093	0.157	0.036	0.126	0.005	0.045	25	1	.080
1	CPV4755	0.200	0.228	0.195	0.286	0.261	0.224	0.176	0.168	0.166	0.064	0.049	.	.	.
2	AP224	0.257	0.214	0.149	0.922	0.204	0.179	0.216	0.253	0.224	0.087	0.095	47	2	.
2	AP239	0.147	0.141	0.130	0.181	0.109	0.240	0.094	0.125	0.219	0.090	0.082	22	0	.
2	AP245	0.203	0.059	0.138	0.246	0.210	0.107	0.180	0.078	0.077	0.107	0.152	28	2	.
2	AP256	0.144	0.048	0.037	0.170	0.122	0.109	0.141	0.139	0.106	0.085	0.085	13	0	.
2	ASA128	0.088	0.146	0.183	0.065	0.266	0.092	0.358	0.174	0.295	0.036	0.022	52	2	.
2	ASA133	0.081	0.179	0.189	0.036	0.238	0.141	0.289	.	0.115	.	0.133	37	3	.005
2	ASA136	0.087	0.149	0.204	0.029	0.390	0.124	0.491	.	0.565	.	0.123	50	.	.
2	ASA137	0.080	0.182	0.099	0.063	0.317	0.170	0.329	0.183	0.149	0.062	0.096	31	0	.
2	ASA141	0.044	0.087	0.097	0.025	0.312	0.082	0.432	.	0.032	.	0.102	28	.	.
2	ASA143	0.044	0.057	0.090	0.025	0.395	0.140	0.357	.	0.475	.	0.029	38	1	.005

2	ASA144	0.070	0.378	0.201	0.055	0.607	0.179	0.488	0.129	0.165	0.080	0.098	23	5	.200
2	ASA147	0.025	0.069	0.110	0.028	0.277	0.109	0.323	.	0.014	.	0.197	.	.	.
2	ASA148	0.052	0.053	0.108	0.028	0.354	0.345	0.613	.	0.292	.	0.137	20	0	.
2	ASA149	0.101	0.209	0.121	0.102	0.364	0.094	0.437	0.280	0.172	0.061	0.052	22	1	.030
2	ASA153	0.151	0.131	0.256	0.038	0.543	0.178	0.394	.	0.053	.	0.173	.	.	.
2	ASA154	0.160	0.064	0.187	0.037	0.310	0.156	0.330	.	0.042	.	0.150	68	2	.115
2	ASA157	0.148	0.200	0.139	0.033	0.025	0.066	0.408	.	.	.	.	21	1	.045
2	ASA160	0.135	0.212	0.112	0.060	0.021	0.072	0.444	0.169	.	0.078	.	.	.	.
2	ASA161	0.058	0.024	0.069	0.027	0.016	0.034	0.284	.	.	.	.	60	1	.
2	ASA162	0.287	0.175	0.107	0.035	0.026	0.121	0.451	.	.	.	.	52	3	.
2	ASA164	0.081	0.221	0.070	0.030	0.023	0.109	0.427	.	.	.	.	31	10	.005
2	ASA165	0.266	0.120	0.119	0.033	0.039	0.075	0.414	.	.	.	.	21	0	1.860
2	ASA166	0.247	0.164	0.132	0.026	0.587	0.063	0.231	.	0.041	.	0.214	25	8	.050
2	ASA168	0.049	0.055	0.084	0.023	0.495	0.048	0.267	.	0.029	.	0.140	68	15	.001
2	ASA169	0.285	0.237	0.201	0.046	0.439	0.052	0.449	.	0.034	.	0.086	13	1	.045
2	ASA170	0.188	0.061	0.165	0.023	0.585	0.040	0.108	.	0.036	.	0.211	22	3	.420
2	ASA172	0.380	0.112	0.159	0.098	0.787	0.089	0.488	0.253	0.035	0.114	0.143	25	10	.030
2	ASA173	0.246	0.040	0.094	0.025	0.404	0.084	0.473	.	0.217	.	0.138	39	0	.020
2	ASA174	0.079	0.063	0.057	0.020	1.052	0.063	0.245	.	0.040	.	0.049	22	6	.
2	ASA175	0.170	0.060	0.064	0.037	0.650	0.050	0.276	.	0.024	.	0.087	21	3	.060
2	ASA179	0.032	0.134	0.205	.	.	0.069	0.347	0.083	0.035	0.215	0.107	22	3	.
2	ASA180	0.045	0.032	0.067	0.032	0.443	0.062	0.308	.	0.029	.	0.106	20	2	.030
2	ASA181	0.164	0.143	0.136	0.233	0.052	0.044	0.074	0.096	.	0.105	.	13	4	.040
2	ASA182	0.085	0.071	0.142	0.120	0.014	0.031	0.029	0.075	.	0.027	.	14	4	.010

2	ASA183	0.172	0.120	0.151	0.222	0.100	0.060	0.261	0.116	0.028	0.100	0.118	46	3	.050
2	ASA184	0.263	0.289	0.186	0.049	0.081	0.046	0.167	.	.	.	.	38	.	.040
2	ASA187	0.298	0.254	0.203	0.141	0.184	0.351	0.277	0.111	0.077	0.187	0.137	18	1	.110
2	ASA190	0.266	0.140	0.123	0.084	0.014	0.024	0.099	0.160	.	0.033	.	28	.	.280
2	ASA195	0.432	0.239	0.227	0.118	0.289	0.038	0.283	0.177	0.438	0.133	0.146	30	2	.020
3	KTPV001	0.062	0.223	0.061	0.087	0.061	0.022	0.382	0.124	.	0.048	.	13	3	.001
3	KTPV002	0.061	0.273	0.069	0.108	0.064	0.026	0.410	0.167	.	0.061	.	27	10	.001
3	KTPV003	0.034	0.248	0.063	0.235	0.340	0.187	0.509	0.134	0.024	0.028	0.042	30	0	.001
3	KTPV004	0.073	0.290	0.088	0.362	0.111	0.414	0.781	0.163	0.208	0.187	0.112	40	3	.050
3	KTPV006	0.040	0.157	0.038	0.241	0.228	0.265	0.538	0.113	0.049	0.142	0.065	26	2	.100
3	KTPV007	0.065	0.530	0.112	0.286	0.208	0.208	0.683	0.202	0.028	0.066	0.071	48	12	.250
3	KTPV013	0.215	0.177	0.031	0.238	0.145	0.285	0.278	0.104	0.007	0.080	0.045	20	0	.090
3	KTPV014	0.021	0.206	0.025	0.083	0.031	0.194	0.228	0.116	.	0.034	.	33	1	.090
3	KTPV016	0.029	0.156	0.066	0.664	0.079	0.132	0.188	0.138	.	0.132	.	18	4	.090
3	KTPV017	0.425	0.404	0.069	0.390	0.552	0.203	0.735	0.143	0.046	0.065	0.041	16	4	.090
3	KTPV018	0.473	0.298	0.135	0.248	0.138	0.130	0.702	0.449	0.015	0.029	0.033	19	5	.030
3	KTPV019	0.252	0.328	0.144	0.200	0.191	0.191	0.914	0.340	0.040	0.027	0.051	39	10	.030
3	KTPV020	0.225	0.165	0.126	0.354	0.665	0.177	0.591	0.320	0.053	0.019	0.049	55	5	.
3	KTPV025	0.211	0.311	0.092	.	.	0.290	0.448	0.304	0.034	0.046	0.057	42	.	.010
3	KTPV026	0.185	0.255	0.049	0.232	0.605	0.123	0.573	0.322	0.062	0.037	0.031	25	5	.030
3	KTPV027	0.247	0.171	0.039	0.211	0.102	0.107	0.337	0.172	0.017	0.020	0.030	38	2	.
3	KTPV028	0.139	0.327	0.081	0.108	0.072	0.099	0.485	0.248	.	0.067	.	23	2	.
3	KTPV029	0.167	0.285	0.062	0.264	0.436	0.082	0.541	0.231	0.007	0.111	0.095	21	4	.
3	KTPV030	0.191	0.232	0.102	0.428	0.350	0.054	0.508	0.279	0.016	0.168	0.107	24	10	.

3	KTPV032	0.278	0.402	0.159	0.343	0.140	0.179	1.277	0.340	0.029	0.026	0.052	45	10	.
3	KTPV036	0.134	0.114	0.123	0.300	0.534	0.089	0.059	0.147	0.019	0.012	0.027	.	3	.030
3	KTPV039	0.183	0.286	0.086	0.388	0.723	0.179	0.214	0.272	0.035	0.020	0.027	49	.	.
3	KTPV040	0.079	0.019	0.064	0.173	0.275	0.044	0.112	0.103	0.005	0.022	0.066	19	.	.
3	KTPV041	0.292	0.173	0.098	0.162	0.263	0.209	0.463	0.174	0.013	0.028	0.035	43	7	.050
3	KTPV042	0.166	0.076	0.097	0.320	0.072	0.185	0.258	0.301	.	0.026	.	23	10	.100
3	KTPV043	0.269	0.120	0.107	0.457	0.794	0.266	0.647	0.132	0.249	0.132	0.073	33	50	.090
3	KTPV044	0.124	0.032	0.032	0.171	0.302	0.058	0.197	0.218	0.010	0.022	0.025	35	20	.090
3	KTPV045	0.128	0.039	0.113	0.126	0.050	0.373	0.312	0.237	.	0.023	.	36	8	.090
3	KTPV046	0.135	0.046	0.144	0.162	0.291	0.105	0.186	0.197	0.008	0.010	0.070	31	10	.
3	KTPV047	0.083	0.039	0.080	0.053	0.047	0.061	0.371	.	.	.	.	38	10	.090
3	KTPV048	0.091	0.044	0.170	0.066	0.052	0.064	0.145	0.203	.	0.010	.	52	10	.
3	KTPV049	0.191	0.051	0.232	0.154	0.343	0.080	0.251	0.224	0.013	0.022	0.084	15	10	.
3	KTPV050	0.130	0.024	0.063	0.157	0.090	0.073	0.097	0.134	0.216	.	.	31	50	.070
3	KTPV054	0.177	0.054	0.130	0.218	0.439	0.096	0.230	0.288	0.119	0.047	.	19	5	.
3	KTPV055	0.199	0.042	0.058	0.074	0.217	0.045	0.164	0.173	.	.	.	37	5	.100
3	KTPV056	0.069	0.017	0.027	0.100	0.032	0.044	0.118	0.192	.	0.029	.	34	3	.
3	KTPV057	0.259	0.309	0.227	0.231	0.218	0.061	0.645	0.260	0.015	0.168	0.095	25	20	.100
3	KTPV058	0.172	0.032	0.030	0.285	0.162	0.046	0.095	0.171	0.055	0.031	0.022	22	.	.100
3	KTPV059	0.155	0.044	0.093	.	0.060	0.133	0.169	0.348	.	0.045	.	50	.	.300
3	KTPV060	0.110	0.036	0.076	0.153	0.238	0.045	0.137	0.181	0.012	0.045	0.015	48	5	.100
3	KTPV064	0.224	0.053	0.205	0.133	0.105	0.084	0.234	0.462	0.283	0.053	0.011	21	2	.020
3	KTPV065	0.124	0.041	0.141	0.111	0.169	0.032	0.216	0.176	0.067	0.028	0.013	35	.	.010
3	KTPV066	0.144	0.047	0.220	0.112	0.072	0.060	0.210	0.361	.	0.058	.	22	3	.010

3	KTPV068	0.032	0.074	0.063	0.414	0.353	0.052	0.221	0.220	0.017	0.082	0.040		16	2	.012
3	KTPV072	0.141	0.112	0.049	0.086	0.062	0.030	0.170	0.212	.	0.048	.		18	.	.012
3	KTPV073	0.019	0.109	0.021	0.071	0.178	0.070	0.153	0.217	0.076	0.038	0.026		15	.	.
3	KTPV076	0.018	0.114	0.017	0.093	0.153	0.067	0.247	0.251	0.020	0.047	0.021		34	10	.
3	KTPV077	0.036	0.244	0.042	0.075	0.347	0.113	0.651	0.333	0.026	0.093	0.040		14	10	.060
3	KTPV078	0.016	0.250	0.030	0.154	0.176	0.052	0.276	0.128	0.168	0.022	0.031		26	10	.005
3	KTPV079	0.006	0.262	0.028	0.080	0.119	0.194	0.293	0.125	0.055	0.032	0.027		16	2	.060
3	KTPV081	0.018	0.211	0.024	0.081	0.144	0.088	0.285	0.280	0.084	0.044	0.018		17	10	.165
3	KTPV083	0.016	0.191	0.027	0.141	0.060	0.072	0.273	0.151	.	0.038	.		45	10	.
3	KTPV088	0.017	0.141	0.030	0.095	0.054	0.062	0.295	0.153	.	0.037	.		60	10	.010
3	KTPV094	0.051	0.140	0.095	0.127	0.462	0.351	0.360	0.258	0.320	0.040	0.029		31	3	.420
3	KTPV096	0.028	0.191	0.040	0.095	0.173	0.185	0.312	0.160	0.024	0.156	0.082		60	10	.
3	KTPV101	0.039	0.196	0.051	0.113	0.267	0.100	0.371	0.271	0.010	0.031	0.023		26	1	.025
3	KTPV103	0.012	0.069	0.016	0.048	0.038	0.089	0.239	.	.	.	.		47	10	.
3	KTPV105	0.018	0.232	0.020	0.044	0.225	0.077	0.233	.	0.015	.	0.013		26	2	.150
3	KTPV106	0.013	0.155	0.028	0.070	0.219	0.060	0.301	0.222	0.008	0.032	0.018		21	8	.
3	KTPV113	0.009	0.273	0.028	0.971	0.041	0.209	0.320	0.337	.	0.043	.		19	10	.
3	KTPV114	0.020	0.203	0.021	0.579	0.051	0.077	0.279	0.155	.	0.030	.		29	2	.100
3	KTPV116	0.013	0.202	0.025	0.338	0.053	0.055	0.277	0.115	.	0.012	.		15	10	.050
3	KTPV117	0.056	0.192	0.050	0.109	0.268	0.110	0.397	0.154	0.056	0.021	0.015		51	6	.020
3	LA03	0.312	0.224	0.241	0.166	0.201	0.287	0.240	0.191	0.012	0.012	0.014		20	1	.170
3	LA04	0.209	0.145	0.228	0.238	0.206	0.355	0.381	0.142	0.011	0.016	0.012		51	10	.030
3	LA05	0.287	0.249	0.198	0.307	0.312	0.495	0.396	0.142	0.021	0.037	0.037		44	2	.010
3	LA08	0.364	0.079	0.063	0.123	0.080	0.207	0.068	0.085	.	0.020	.		22	1	.040

[1] NPI – Number of previous malaria infections
[2] P - Parasitemia

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