UNIVERSITY OF CALIFORNIA, SAN DIEGO

SAN DIEGO STATE UNIVERSITY

MALARIA IN THE PERUVIAN AMAZON

Submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Public Health (Global Health)

by

Raul Chuquiyauri

Committee in charge:

University of California, San Diego

Professor Joseph Vinetz, Chair
Professor Kimberly Brouwer
Professor Richard Garfein

San Diego State University

Professor Stephanie Brodine
Professor John Weeks

2014

The Dissertation of Raul Chuquiyauri is approved, and is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

_____

_____

_____

_____

University of California, San Diego

San Diego State University

2014

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Chapter 2

Chapter 3

ACKNOWLEDGEMENTS

I would like to acknowledge Professor Joseph Vinetz for his support as the chair of my committee. Through multiple drafts and many years working together, his guidance has proved to be invaluable.

Chapter 2, in full, is a reprint of the material as it appears in Acta Tropica, 2012, March; 121(3): 292-302. The dissertation author was the primary investigator and author of this paper. I would like to acknowledge co-authors Maribel Paredes, Pablo Penataro, Sonia Torres, Silvia Marin, Alexander Tenorio, Kimberly Brouwer, Shira Abeles, Alejandro Llanos, Robert Gilman, and Margaret Kosek, for their contribution to this paper and permission to use it as part of my dissertation.

Chapter 3, in full, is a reprint of the material as it appears in Am J. Trop. Med. Hyg., 89(2), 2013, pp. 326-338. The dissertation author was the primary investigator and author of this paper. I would like to acknowledge co-authors Pablo Penataro, Kimberly Brouwer, Manuel Fasabi, Maritza Calderon, Sonia Torres, Robert Gilman, and Margaret Kosek, for their contribution to this paper and permission to use it as part of my dissertation.

Chapter 4, in full, is a reprint of the material as it is being prepare for submission to PLoS Journal. The dissertation author was the primary investigator and author of this paper. I would like to acknowledge co-authors Douglas Molina, Huw Davies, Eli Moss, Ruobing Wang, Malcolm Gardner, Kimberly Brouwer, Sonia Torres, Robert Gilman, Hugo Rodriguez, Alejandro

Llanos, Daniel Neafsey, and Philip Felgner, for their contribution to this paper and permission to use it as part of my dissertation.

VITA

2001 Bachelor of Medicine, Cayetano Heredia Peruvian University, Lima, Peru.

2002 Medical Doctor, Cayetano Heredia Peruvian University, Lima, Peru.

2006 Tropical Medicine, Johns Hopkins Bloomberg School of Public Health, Baltimore, US.

2013 Post-Doc, University of California Global Health Institute, US.

2014 Master of Public Health, San Diego State University, San Diego, US.

2014 Doctor of Philosophy, University of California San Diego, San Diego State University, San Diego, US.

PUBLICATIONS

Moreno M, Tong C, Guzman M, ***Chuquiyauri R,*** Llanos-Cuentas A, Rodriguez H, Gamboa D, Meister S, Winzeler EA, Maguina P, Conn JE, Vinetz JM. Infection of Laboratory-Colonized *Anopheles darlingi* Mosquitoes by *Plasmodium vivax. Am. J. Trop. Med. Hyg.* 2014 Feb 17.

Chuquiyauri R, Peñataro P, Brouwer KC, Fasabi M, Calderon M, Torres S, Gilman RH, Kosek M, Vinetz JM. Microgeographical differences of *Plasmodium vivax* relapse and reinfection in the Peruvian Amazon. Am J Trop Med Hyg. 2013 Aug;89(2):326-38.

Abeles S, ***Chuquiyauri R***, Tong C, and Vinetz JM. Human host-derived cytokines associated with *Plasmodium vivax* transmission from acute malaria patients to Anopheles darlingi mosquitoes in the Peruvian Amazon. *Am. J. Trop. Med. Hyg*. 2013 Mar 11.

Bright AT, Tewhey R, Abeles S, ***Chuquiyauri R***, Llanos-Cuentas A, Ferreira MU, Schork NJ, Vinetz JM, and Winzeler EA. Whole genome sequencing analysis of *Plasmodium vivax* using whole genome capture. *BMC Genomics,* 2012: 13:262.

Kosek M, Yori PP, Gilman RH, Calderon M, Zimic M, ***Chuquiyauri R,*** Jeri C, Pinedo-Cancino V, Matthias MA, Llanos-Cuentas A, Vinetz JM. High degree of *Plasmodium vivax* diversity in the Peruvian Amazon demonstrated by tandem repeat polymorphism analysis. *Am. J. Trop. Med. Hyg*., 86(4), 2012, pp. 580-586.

Chuquiyauri R, Paredes M, Peñataro P, Torres S, Marin S, Tenorio A, Brouwer KC, Abeles S, Llanos-Cuentas A, Gilman RH, Kosek M, Vinetz JM. Socio-demographics and the development of malaria elimination strategies in the low transmission setting. *Acta Trop*. 2012 Mar;121(3):292-302.

Bounkeua V, Li F, ***Chuquiyauri R***, Abeles S, McClean C, Neyra V, Llanos-Cuentas A, Yori PP, and Vinetz JM. Lack of molecular correlates of *Plasmodium vivax* ookinete development. *Am. J. Trop. Med. Hyg*., 85(2), 2011, pp. 207-213.

Chuquiyauri R, Vilcarromero S, and Rodriguez H. Peruvian Health System, 2010. Chapter Supplement in: Comparative Health Systems: Global Perspectives for the 21st Century, by James A. Johnson and Carleen H. Stoskopf; Jones & Bartlett Publishers.

Van Deun A, ***Chuquiyauri R***, Torrea G, Agapito JC, Verdonck K, and Gotuzzo E. Yield of fluorescence microscopy versus culture for tuberculosis at a middle-income country referral hospital. Trans R Soc Trop Med Hyg., 132(6), 2008, pp. 564-569.

Bharti AR, Patra KP, ***Chuquiyauri R***, Kosek M, Gilman RH, Llanos-Cuentas A, and Vinetz JM. Polymerase chain reaction detection of *Plasmodium vivax* and *Plasmodium falciparum* DNA from stored serum samples: implications for retrospective diagnosis of malaria. *Am J Trop Med Hyg,* 77(3), 2007, pp. 444-446.

Bharti AR, ***Chuquiyauri R***, Brouwer KC, Stancil J, Lin J, Llanos-Cuentas A, and Vinetz JM. Experimental infection of the neotropical malaria vector *Anopheles darlingi* by human patient–derived *Plasmodium vivax* in the Peruvian Amazon. *Am. J. Trop. Med. Hyg*., 75(4), 2006, pp. 610-616.

Zamudio C, Seas C, Hernandez K, Ramos E, Verdonck K, ***Chuquiyauri R***, Echevarria J, Legua P, Maguiña C., and Gotuzzo E. Morbilidad y mortalidad en el servicio de hospitalización del Departamento de enfermedades infecciosas, tropicales y dermatológicas del Hospital Nacional Cayetano Heredia entre 1990 - 2000. *Rev Med Hered*., 15(4), 2004, pp. 181-187.

Chuquiyauri R, Verdonck K, Gonzalez E; Zamudio E, Echevarria J, Seas C and Gotuzzo E. Morbi–mortalidad de pacientes con tuberculosis

hospitalizados en el departamento de enfermedades infecciosas, tropicales y dermatológicas del Hospital Nacional Cayetano Heredia, Lima - Perú entre los años 1990 y 2000. *Rev Med Hered.*, 15(4), 2004, pp. 203-210.

ABSTRACT OF THE DISSERTATION

MALARIA IN THE PERUVIAN AMAZON

by

Raul Chuquiyauri

Doctor of Philosophy in Public Health (Global Health)

University of California, San Diego 2014

San Diego State University, 2014

Professor Joseph Vinetz, Chair

Malaria is the most widespread serious parasitic disease worldwide, with over 2 billion people at risk of infection, over 300 million cases yearly, and over 1 million deaths every year. The primary vector in the Peruvian Amazon, *Anopheles darlingi,* is a highly anthropophilic rural mosquito. This thesis research focuses primarily on *Plasmodium vivax* malaria in the Peruvian

Amazon, for three reasons: it is the most common form of malaria in the Peruvian Amazon; in comparison with *Plasmodium falciparum, Plasmodium vivax* malaria is understudied in this region; and the current malaria elimination call to action leads the need for new knowledge on this disease in the Peruvian Amazon. The goal of this thesis research is to contribute with the elimination of malaria transmission by means of testing the overarching central hypothesis that sociodemographics, relapsing *Plasmodium vivax* malaria, and the use of protein microarray analysis, are current aspects/tools that will help to stopping transmission of *Plasmodium vivax* malaria within rural villages near Iquitos, and eventually within the Amazon Region of Loreto, Peru. Three studies were conducted and published for this purpose.

The first published manuscript analyzes socio-demographical and occupational activities of people at risk for malaria. This analysis allowed us to identify the main risk group among subjects working in the forest or agricultural activities and their partners at home. The second manuscript demonstrated new tools for malaria epidemiology studies: tandem repeat markers and restriction fragment length polymorphism msp3α markers that have high discriminatory capacity for determining the micro-geography of malaria transmission in the Peruvian Amazon. The third manuscript used genome-level protein microarray analysis to identify new antigens as serological tool to monitor infection status and transmission dynamics, and that proteins recognized by human IgG antibodies are more likely to have amino acid changes or single nucleotide polymorphisms than proteins coded by the

genome as a whole, suggesting selection arising from host-pathogen interactions, immune or otherwise. The new *P. vivax* protein antigens have important potential as tools for sero-epidemiological analysis of surveillance, control and elimination strategies. More generally, these research in the Peruvian Amazon contribute to a better understanding of biological and socio-demographical characteristics of malaria transmission and fundamental properties of the human immune response to *Plasmodium vivax.*

CHAPTER 1. INTRODUCTION

The protozoal agents of malaria are blood parasites members of the genus *Plasmodium*. *Plasmodium* species are members of the phylum Apicomplexa, and have a life cycle involving a vertebrate host and arthropod vector.[1,2,3] Currently, there are approximately 156 named species of *Plasmodium* which infect various species of vertebrates. *Plasmodium* species are host and vector specific meaning that each species will only infect a limited range of hosts and vectors.[4] Five species parasitize humans, as they utilize humans as a natural intermediate host: *P. falciparum, P. vivax, P. ovale, P. malariae*, and the zoonotic *P. knowlesi.*[5,6]

Malaria Parasite Life Cycle. The malaria parasite life cycle (Fig. 1.1) involves two hosts. While ingesting a blood meal, a Plasmodium-infected female *Anopheles* mosquito definitive host inoculates saliva along with sporozoites into the human intermediate host that, within about 30-60 minutes, invade hepatic cells and mature into schizonts, that 7-10 days later rupture and release merozoites.[2,3,7,8] In *P. vivax* and *P. ovale,* a dormant stage called hypnozoites persist in the liver and cause relapse by invading the bloodstream months, or even years later. One sporozoite infected hepatocyte develops into around 10000 to 30000 merozoites during this initial replication within a hepatic cell (exo-erythrocytic schizogony). Then, parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony); the ring stage

trophozoites mature into schizonts, which rupture releasing merozoites.[3,8] A small proportion of parasites differentiate into sexual erythrocytic stages (gametocytes), that are the infective form for mosquitoes.[9,10] Blood stage parasites are responsible for the clinical manifestations of the disease. Gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal. The parasites' multiplication in the mosquito is known as the sporogonic cycle. While in the mosquito's stomach, a microgamete penetrates a macrogamete generating a zygote. A zygote in turn becomes motile and elongated (ookinete) to invade the midgut wall of the mosquito where to develop into an oocyst. An oocyst grows, ruptures, and releases sporozoites, which make their way to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host continues the malaria life cycle.[2,3,8]

Malaria Brief History. It is probable that natural selection of human plasmodial species took place in the Pleistocene.[11-13] Human ancestors lived in close contact with simian species in Africa and Asia.[11] Some anopheline mosquitoes of the Oligocene period around 30 million years ago adapted themselves to feeding on the blood of early hominids and Neolithic dwellers, including early Chinese and Greeks.[14] Ancient documents testify to malaria's long time ago widespread distribution within human populations, such Indian writings of the Vedic period (1500 to 800 BC) where malaria is regarded as the "king of diseases".[15] In 270 BC, enlargement of the spleen was described as a

sign in malaria by the Chinese medical canon known as the *Nei Ching.*[11] To mention a few of the historical writings on malaria along history, we had: *The Illiad from* Greek poet Homer (750 BC)*, The Wasps* from Aristophanes (445-385 BC)*,* and the writings from Aristotle (384-322 BC), Plato (428-347 BC), and Sophocles (496-406 BC).[16] In the fifth century BC, Hippocrates observed the relationship between the appearance of malaria and the seasons of the year and was the first to describe the clinical picture of malaria, including periodic fevers.[8] Celsus (25 BC - 54 AD) gave description of the various types of malaria.[8]

Malaria came to Europe from Africa by way of the Nile valley, and caused epidemics in crowded settlements, such as the one 79 AD that destroyed the croplands surrounding Rome, pushing local farmers to abandon their fields[17] and to remain sparsely settled until malaria was eliminated in the late 1930s.[8,17] There are writings describing how the development of China's south lagged behind its north due to higher an unequal burden of malaria around the Ganges valley to the south.[8] *Plasmodium malariae* and *Plasmodium vivax* were carried to The Americas by European invaders and colonists.[16]

In The Americas, malaria contributed to poor physical and economic health on the entire region, and exerted its deleterious effect in the United States until the hydroelectric power and modernization was setup in the South during the 1930s. Malaria reclaimed American's attention during World War II when more soldiers fell to malaria than to enemy forces, and the modern

Centers for Disease Control and Prevention was founded because of malaria.[3] Starting the 17th century the value of the "Peruvian" bark for the treatment of fevers was discovered; and during the 18th century, these specific fevers received the Italian name "malaria", because it was then widely believed that their cause was related to the foul air common near marshy areas. In 1735, the tree producing the Peruvian bark was given its scientific name of *Cinchona* by Linnaeus. Quinine was isolated in 1820 by Pelletier and Caventou in France,[3] who released it into the public domain for the benefit of mankind. Since Europeans do not possess the genetic resistance to severe malaria found in native African populations, quinine enabled Europeans to explore and settle in Africa.[10] A strong testimony of the greatest burden of malaria in Africa is the inherited disorder sickle cell anemia which give at least 90% protection against the mortality of malarial infection.[3,8] In some parts of Africa, more than 30% of the population carry a single copy of the abnormal gene.[14,18-23]

In 1880, a French surgeon in Algeria, Alphonse Laveran, discovered the parasitic agent of malaria, a milestone in the history of medicine.[24] Soon after that, Romanowsky in Russia developed a new method of staining the malaria parasites in blood films. In 1897, Ronald Ross elucidated the actual mode of transmission by a mosquito bite that had previously fed on an infected patient with the plasmodia in his blood.[11] At this time, malaria was entrenched along the Mediterranean and its transmission increased dramatically in the wake of wars and major social and economic upheavals.[11] Malaria vector control by targeting the *Anopheles* vector during its aquatic larval stage was

initiated by Ross at the beginning of the 20th century. The whole complex picture of the cycle of development of malaria parasites in humans and in the female *Anopheles* mosquito became clear with studies by the Italians Amico Bignami, Giuseppe Bastianelli, and Battista Grassi in 1899. The existence of the exo-erythrocytic phase of human malaria was discovered in 1951 by Shortt and Garnham.[7,12]

The residual action of dichloro-diphenil-trichloroethane (DDT) was discovered by Paul Muller in Switzerland in 1939 and the possibilities of malaria eradication became closer to reality.[11] In 1955 the World Health Organization (WHO) adopted the achievement of the global malaria eradication as its goal and in 1956 the Expert Committee of Malaria of the WHO prepared the basic guidelines for this international endeavour and malaria was eliminated as a major health problem in much of the world in 1963.

Other valuable synthetic drugs developed by the Germans, the Frenchs, the Americans, and the Britains followed in 1934 (Chloroquine), 1944 (Proguanil), 1946 (Amodiaquine), 1950 (Primaquine), and 1952 (Pyrimethamine). Malaria plagued the French and British when engaged in battle during World War I and II. Again, during the Korean and Vietnam wars, malaria was a "The Great Debilitator", with *Plasmodium vivax* causing the greater morbidity in the former World War I, and *Plasmodium falciparum* causing the greater morbidity in the latter World War II.

Global importance of malaria. Annually, there are an estimated 135-287 million cases of malaria;[25] malaria deaths have increased from an estimated 995,000 in 1980 to 1'817,000 in 2004, and 1'238,000 in 2010,[26] primarily children in sub-Saharan Africa.[15,26] These numbers will double over the next 20 years without effective control. Although the effects of repeated attacks of *P. vivax* are rarely directly lethal, they have major deleterious effects on well-being, growth, and development, and on the economic performance at the individual, family, community, and national levels. *P. vivax* malaria is a major cause of morbidity and leading to a significant impact on the quality of life for more than 1.5 to 2.5 billion people at risk, and with 83 million disability-adjusted life years lost in 2010.[26-32] Humans are the only known reservoir for *P. falciparum* and *P. vivax;* these parasites have no known animal reservoir. The 100 million malaria cases occurring yearly outside of Africa are, in roughly equal proportions, due to *P. falciparum* or *P. vivax.* Indeed, all malaria endemic regions outside of Africa, and in some places within Africa, these parasite species co-exist.

It is virtually impossible, using drugs alone, to ensure that a malaria-infected individual has received the necessary treatment at the time at which she or he is infectious to mosquitoes. The development and deployment of vaccines designed to limit the transmission of human malaria infections to mosquitoes – malaria transmission blocking vaccines – are crucial. There has been a recent explosion of information from large scale genomic, proteomic and gene expression profiling of multiple *Plasmodium* species. The whole

genome sequence of *Homo sapiens* and the major malaria-transmitting vector mosquito, *Anopheles gambiae* have been published. A major challenge confronting the malaria field is how to apply such knowledge towards the amelioration of malaria at the field level, in the real world setting. One such approach is to apply the genomic information towards the discovery of and validation of stage-specific targets of interfering with *Plasmodium* biology, which is expected to lead to new, more effective, drugs and vaccines.

The relevance of *P. vivax* biology to understanding its epidemiology and transmission. In the rural areas of the Amazon Region, around 15-25% of the population works on agriculture, logging, and harvesting wood for charcoal and because of these activities they usually travel outside the natural borders of their villages; these people is serving as a source for introducing and re-introducing *P. vivax* to their home villages and would be acting as reservoirs of continuing malaria transmission. Therefore, they are prime targets against which to deploy malaria transmission-blocking strategies.

P. vivax generally causes uncomplicated malaria characterized by generalized signs and symptoms such as fever, headache, chills, sweats, splenomegaly, and anemia; occasionally, complicated disease occurs that may result in pulmonary compromise, secondary bacterial infection, spleen rupture and even death.[3,8] Drug treatment of acute vivax malaria that targets asexual erythrocytic stages does not affect gametocytes so that transmission to mosquitoes may continue even after the malarial episode is treated.

Another important characteristic is that in *P. vivax* infection, infectious gametocytes appear simultaneously with asexual stages in the blood, so that people can serve as sources for infecting mosquitoes early in the course of disease. In *P. falciparum* malaria, gametocytes appear late, after several weeks, in the presence of clinical immunity, which serves to limit transmission if the malarial episode is treated quickly.

In the pre-erythrocytic stage of infection, a key difference with *P. falciparum* is that in *P. vivax* infection, dormant hepatocyte forms develop. These are called hypnozoites and can result later in blood stage infection and symptoms of malaria, termed ‘relapsing malaria’. Anti-malarial drugs directed against asexual blood stages, such as chloroquine, do not affect liver forms, so that to prevent relapse, specific chemotherapy is required; the only drug available for this purpose is primaquine. Standard treatment for *P. vivax* in Peru is chloroquine plus primaquine, provided free from the Ministry of Health. The relapse of *P. vivax* from liver forms is epidemiologically important because humans with hypnozoites are reservoirs of re-initiating and maintaining vivax transmission. This mechanism is also important for *P. vivax* to be able to continue transmission between malaria seasons.

Malaria transmission is highly localized, transmitted within focal areas of human populations in which infected people come into contact with anthropophilic anopheline mosquitoes. Mosquito breeding and feeding habits put constraints on their capacity to transmit malaria: they are limited to a close distance from their breeding sites and they must gain access to human

dwellings at night. Given this situation, any means that can reduce malaria transmission-whether application of larvicides, residual insect spraying, drainage, or transmission blocking vaccines-can be used in local contexts as small as one-quarter to one-half mile. Vector control, by any of these means, has historically been the only effective means of malaria control but this depends on continuing anti-mosquito abatement programs, difficult to maintain politically and ecologically on a long-term basis. Furthermore, the sexual stages of the parasite's life cycle are the only ones which are extracellular and thus accessible to antibodies and complement from the human immune system (through passive immunization of mosquitoes), for more than a few seconds or minutes (which is the case for pre-erythrocytic and blood asexual stages) – in this case for up to 24 hours. Despite no immediate role in personal protection, TBVs are a potentially powerful component of a multifaceted public health approach to controlling or eliminating malaria. At the community level, an effective malaria TBV could have an impact analogous to the role that vaccines such as smallpox, polio, and measles have had in the eradication of their respective diseases by preventing the transmission of pathogens from individuals to the community. An effective transmission-blocking vaccine may extend the useful life of newly developed drugs or other types of malaria vaccines by reducing the passage of mutant parasites from drug- or vaccine-treated humans to mosquitoes.

***Plasmodium vivax* malaria and infection: relation to epidemiology and transmission.** *P. vivax* generally causes uncomplicated malaria characterized by generalized signs and symptoms such as fever, headache, chills, sweats, splenomegaly and anemia; occasionally, complicated disease occurs that may result in pulmonary compromise, secondary bacterial infection, spleen rupture and even death. Despite rarely being lethal, vivax malaria is profoundly debilitating and takes an enormous human and economic toll. We and others have found that neither *P. falciparum* nor *P. vivax* is more likely to produce a state of asymptomatic parasitemia, at least in the Amazon region. Drug treatment of acute malaria that targets asexual erythrocytic stages may not affect gametocytes (except for Artesunate derivatives)—indeed may increase the number of circulating gametocytes as has been shown with chloroquine and sulfadoxine/pyrimethamine—so that transmission to mosquitoes may continue even after the malarial episode is treated. Another important difference between *P. vivax* and *P. falciparum* is that in *P. vivax* infection, infectious gametocytes appear simultaneously with asexual stages in the blood, so that people can serve as sources for infecting mosquitoes early in the course of disease. In *P. falciparum* malaria, gametocytes appear late, after several weeks, in the presence of clinical immunity, which serves to limit transmission if the malarial episode is treated quickly.

Anti-malarial drugs directed against asexual blood stages, such as chloroquine, do not affect liver forms, so that to prevent relapse, specific chemotherapy is required; the only drug available for this purpose is

primaquine. Standard treatment for *P. vivax* in Peru is chloroquine plus primaquine, provided free from the Ministry of Health (although compliance is not monitored closely and the regimen frequently is not completed). The relapse of *P. vivax* from liver forms is epidemiologically important because humans with hypnozoites are reservoirs of re-initiating and maintaining vivax transmission. This mechanism is also important for *P. vivax* to be able to continue transmission between malaria seasons, especially as asymptomatic *P. vivax* relapse is common.[33] However, an important gap in malaria knowledge is whether *P. vivax* relapse or other mechanisms contribute more to maintaining *P. vivax* circulation within a given human population.

Overarching central hypothesis. *Plasmodium vivax* malaria is endemic in the Amazon Region of Loreto, Peru. The long-term goal of this thesis research is to contribute towards the elimination of malaria transmission. This thesis research seeks to test the overarching central hypothesis that human behavior, mosquito ecology, and *P. vivax* biology (relapsing *P. vivax* malaria), combine to maintain malaria transmission. The development of new tools to asses subclinical infections and the regional movement of *Plasmodium vivax* parasites related to human behavior will be key to elimination strategies in rural villages near Iquitos, and eventually within the Amazon Region of Loreto, Peru. The proposed research integrates molecular relapsing aspects of *P. vivax*, sociodemographics, and microarrays analysis, a comprehensive approach that will be critical for success of malaria interventions. This thesis

research focuses on malaria in the Peruvian Amazon, specially on *P. vivax,* for three reasons: 1) As in most Panamerican Health Organization (PAHO) member states and countries outside of Africa, it is the most common form of malaria in the Peruvian Amazon, with substantial morbidity representing a significant public health threat; 2) In comparison with *P. falciparum, P. vivax* malaria is understudied; and 3) The current malaria elimination call to action leads to shrinking the malaria map switching from control to elimination strategies, therefore, increasing the need for new knowledge on this disease in the Peruvian Amazon.

This doctoral thesis research is focused on malaria in the Peruvian Amazon and is novel in that it will provide highly defined insights into the relevance of sociodemographics and occupational exposures, the importance of differentiating recurrent *P. vivax* infections (relapses versus reinfections), and the first insights into protein microarray analysis as a tool for establishing tools for understanding antibody decay kinetics as the basis for applying sero-epidemiology to surveillance, control, and elimination. Results from these studies will be applicable to the Amazon region and other regions around the world with similar transmission dynamics; and this knowledge is essential for further advancement in human global fight against malaria.

The publication described in chapter 2 tests a central hypothesis that a major factor responsible for maintaining the transmission of *P. vivax* within rural villages near Iquitos, Peru is, that people who travel outside of their home villages become infected with *P. vivax*, and have a increased malaria number

of episodes lifetime; such people would be a key population to target with a malaria transmission-blocking vaccine to prevent parasite reintroductions. Similarly, migrants might have the same risk factor for introducing new strains of *Plasmodium* into a village. The socio-demographic features of people living in the peri-Iquitos region, particularly the frequency of rural travel outside of villages for agricultural-related activities, suggests that travelers (or incoming migrants) are important for maintaining *P. vivax* in these human populations.

The chapter 3 paper will test the hypothesis that recurrences caused by relapse are less common than recurrences caused by reinfection. In addition, we explore if a higher level of differentiating *P. vivax* infecting strains during initial and subsequent infections could be improved by comparing PvMSP-3a PCR-RFLP versus TR polymorphism analysis in parasites from an initial versus a subsequent *P. vivax* infection, hence allowing relapses to be distinguished from new infections. Such analysis is the key to understanding the transmission dynamics and role of human movement in the maintenance and spread of *P. vivax* in endemic regions.

The chapter 4 paper will test the central hypothesis that there is boosting in some antigens when we have recurrent infections due to relapses, as compared with reinfections. This knowledge is of great importance in the further identification and development of new tools for serologic surveillance and vaccine discovery. We expect this thesis research to provide key parasitological, epidemiological, and sociodemographical information essential

for establishing field sites for testing malaria transmission-blocking vaccines, which by necessity will need to be deployed on a micro-environmental scale.

REFERENCES

1. Harrison, G. 1978. *Mosquitoes, Malaria, and Man: A History of the Hostilities Since 1880.* New York: Dutton.

2. Fairhurst RM, Wellems TE. Plasmodium species (Malaria). In: Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases, 6th edition. Elsevier Inc. Chapter 272:3121-3144.

3. Gilles HM, Warrel DA. Bruce Chwatt's Essential Malariology. 4th ed. New York: Oxford University Press, 2002.

4. Bruce-Chwatt LJ. Malaria as a zoonosis. WHO/Zoon/66.90; WHO/Mal/66. 578. 1966.

5. Singh B, Sung LK, Matusop A, Radhakrishnan A, Shamsul SS, Cox-Singh J, Thomas A, Conway DJ. 2004. A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. *Lancet* 363(9414):1017–1024.

6. Cox-Singh J, Davis TM, Lee KS, Shamsul SS, Matusop A, Ratnam S, Rahman HA, Conway DJ, Singh B. 2008. *Plasmodium knowlesi* malaria in humans is widely distributed and potentially life threatening. *Clin Infect Dis* 46(2): 165–171.

7. Shortt HE, Garnham PCC. The pre-erythrocytic development of *Plasmodium cynomolgi* and *Plasmodium vivax. Trans R Soc Trop Med Hyg*. 1948;41:785-795.

8. White NJ, Breman JG. Malaria. In: Harrison's Principles of Internal Medicine. 18th ed. Mc Graw Hill, 2011, 1280-1293.

9. Ross R. On some peculiar pigmented cells found in two mosquitoes fed on malarial blood. *Br Med J*. 1897; 1786-1788.

10. Grassi B. Rapporti tra la malaria e peculiari insetti. Atti R Accad Lincei. 1898;7:163-172.

11. Bruce-Chwatt, De Zulueta. 1988. The rise and fall of malaria in Europe. Oxford University Press.

12. Garnham. 1966. Malaria parasites and other Haemosporidia. *Science,* vol. 157, No. 3792, 1132pp.

13. Mattingly PF. 1976. Evolution of the malarias: the problem of origins. Parassitologia 18(1-3):1-8.

14. Carter R, Mendis KN. 2002. Evolutionary and historical aspects of the burden of malaria. *Clinical Microbiology Review* 15(4):564-594.

15. Miller LR, Ikram S, Armelagos GJ, Walker R, Harer WR, Shiff CJ, Baggett D, Carrigan M, Maret SM. 1994. Diagnosis of Plasmodium falciparum infections in mummies using rapid manual ParaSight-F test. *Tran R Soc Trop Med Hyg.,* 88(1):31-32.

16. Sherman IW. 1998. A brief history of malaria and discovery of the parasite's life cycle. In: Sherman IW, ed. *Malaria: Parasite Biology, Pathogenesis and Protection.* Washington, DC: ASM.

17. Cartwright F. 1991. *Disease and history.* New York: Dorset Press.

18. Allison AC. 1965. Population genetics of abnormal haemoglobins and glucose-6-phosphate dehydrogenase deficiency, p 365-391. *In* JHP Jonxis (ed.), Abnormal haemoglobins in Africa. Blackwell, London, United Kingdom.

19. Cavalli-Sforza LL, Menozzi P, Piazza A. 1994. The history and geography of human genes. Princeton University Press, Princeton, NJ.

20. Livingstone FB. 1967. Abnormal haemoglobins in human populations. Aldine, Chicago, Ill.

21. Livingstone FB. 1971. Malaria and human polymorphisms. *Annu. Rev. Genet*. 5:33-64.

22. Hill AVS, Catherine EM, Kwiatkowski D, Anstey NTM, Twumasi P, Rowe PA, Bennett S, Brewster D, McMichael AJ, Greenwood BM. 1991. Common West African HLA antigens are associated with protection against severe malaria. *Nature* 352:595-600.

23. Bayoumi RA. 1987. The sickle-cell trait modifies the intensity and specificity of the immunes response against *P. falciparum* malaria and leads to acquired protective immunity. *Med Hypotheses,* 22287-298.

24. Laveran CL. 1982. Classics in infectious diseases: A newly discovered parasite in the blood of patients suffering from malaria. Parasitic etiology of attacks of malaria: Charles Louis Alphonse Laveran (1845-1922). *Rev Infect Dis.* 1982; 4(4):908-911.

25. WHO. World Malaria Report 2013. Geneva: World Health Organization, 2013.

26. Murray CJL, Rosenfeld LC, Lim SS, And*rews KG,* Foreman KJ, Haring D, Fullman N, Naghavi M, Lozano R, Lopez AD. 2012. Global malaria mortality between 1980 and 2010: a systematic analysis. *Lancet,* vol. 379:413-431.

27. Sachs J, Malaney P. The economic and social burden of malaria. *Nature*. 2002; 415(6872):680-685.

28. Breman JG. The ears of the hippopotamus: manifestations, determinants, and estimates of the malaria burden. *Am J Trop Med Hyg*. 2001; 64(1-2 Suppl):1-11.

29. Institute of Medicine. Malaria: Obstacles and opportunities. Oaks SC, Mitchell VS, Pearson GW, Carpenter CCJ, eds. Washington DC: National Academy Press, 1991

30. Tusting LS, Willey B, Lucas H, Thompson J, Kafy HT, Smith R, Lidsay SW. Socioeconomic development as an intervention against malaria: a systematic review and meta-analysis. *Lancet.* 2013; 382(9896):963-972.

31. Gething PW, Patil AP, Smith DL, Guerra CA, Elyazar IR, Johnston GL, Tatem AJ, Hay SI. A new world malaria map: *Plasmodium falciparum* endemicity in 2010. *Malar J.* 2011;10:378.

32. Gething PW, Elyazar IR, Moyes CL, Smith DL, Battle KE, Guerra CA, Patil AP, Tatem AJ, Howes RE, Myers MF, George DB, Horby P, Wertheim HF, Price RN, Müeller I, Baird JK, Hay SI. A long neglected world malaria map: *Plasmodium vivax* endemicity in 2010. *PLoS Negl Trop Dis*. 2012;6(9):e1814.

33. Van den Eede P, Soto-Calle VE, Delgado C, Gamboa D, Grande T, Rodriguez H, Llanos-Cuentas A, Anne J, D' Alessandro U, Erhart A. *Plasmodium vivax* sub-patent infections after radical treatment are common in Peruvian patients: results of a 1-year prospective cohort study. *PLoS One* 28;6(1):e16257.

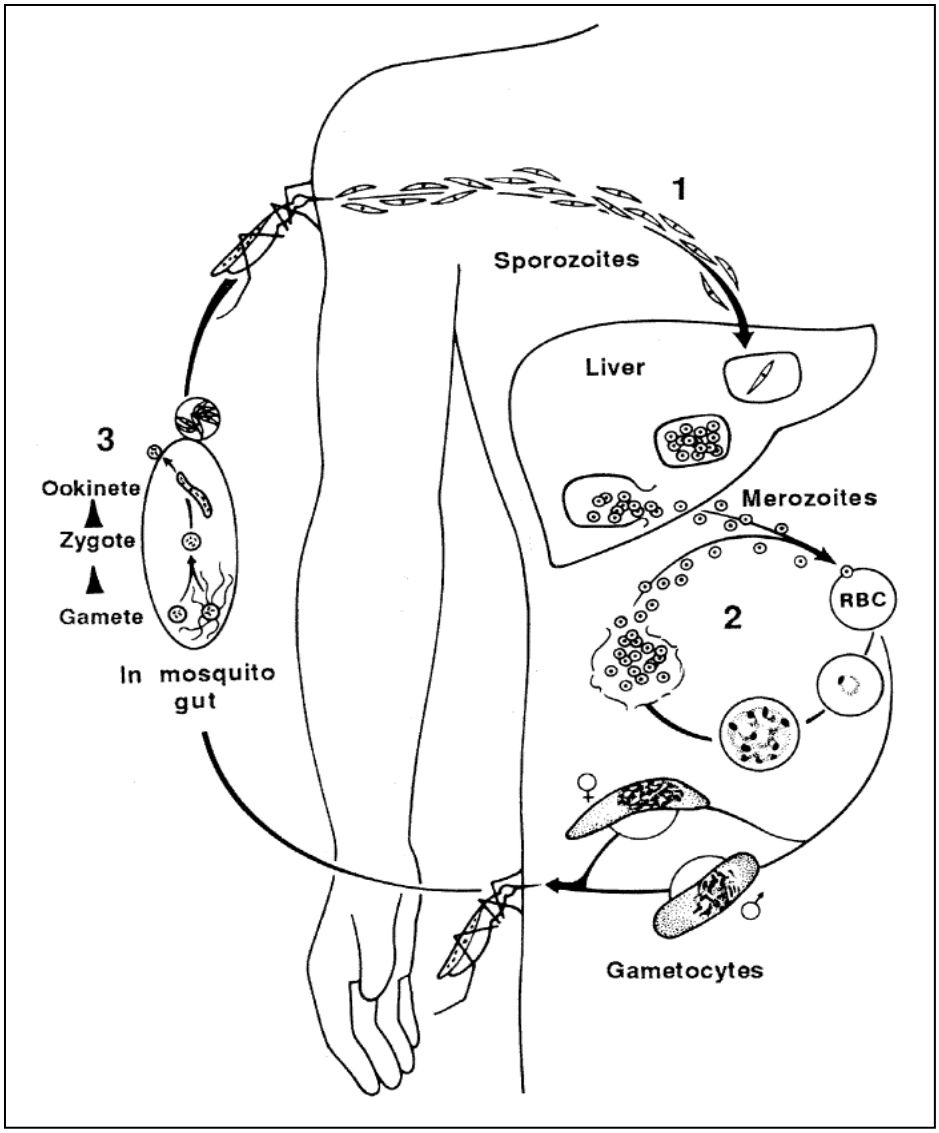

Figure 1.1. Malaria Parasite Life Cycle. (Source: Miller et al, *Science* 1986)

CHAPTER 2: SOCIO-DEMOGRAPHICS AND THE DEVELOPMENT OF MALARIA ELIMINATION STRATEGIES IN THE LOW TRANSMISSION SETTING

ABSTRACT

This analysis presents a comprehensive description of malaria burden and risk factors in Peruvian Amazon villages where malaria transmission is hypoendemic. More than 9,000 subjects were studied in contrasting village settings within the Department of Loreto, Peru, where most malaria occurs in the country. *Plasmodium vivax* is responsible for more than 75% of malaria cases; severe disease from any form of malaria is uncommon and death rare. The association between lifetime malaria episodes and individual and household covariates was studied using polychotomous logistic regression analysis, assessing effects on odds of some vs. no lifetime malaria episodes. Malaria morbidity during lifetime was strongly associated with age, logging, farming, travel history, and living with a logger or agriculturist. Select groups of adults, particularly loggers and agriculturists acquire multiple malaria infections in transmission settings outside of the main domicile, and may be mobile human reservoirs by which malaria parasites move within and between micro-regions within malaria endemic settings. For example, such individuals might well be reservoirs of transmission by introducing or reintroducing malaria into their home villages and their own households, depending on vector ecology and the local village setting. Therefore, socio- demographic studies can

identify people with the epidemiological characteristic of transmission risk, and these individuals would be prime targets against which to deploy transmission blocking strategies along with insecticide treated bednets and chemoprophylaxis.

INTRODUCTION

Global malaria control efforts have increased massively in recent years to levels not seen since eradication efforts in much of the world commenced after World War II.[1] The development of sustainable local malaria control measures, regional elimination programs, and ultimately the holy grail of global eradication, is predicated on the notion that the prevalence of parasitemia in human populations can be effectively monitored and control measures evaluated over space and time.[2,3] This static notion of malaria control presupposes forward progress in malaria control, but does not necessarily account for local or regional reintroductions by mobile human reservoirs of malaria that may travel within or between endemic regions for economic, social or other behavior reasons. Here we will suggest that one reason for malaria's intractability is that human behavior allows malaria parasites to move among regions with anophelism thus allowing this disease to maintain its hold on human populations.

In Latin America and the Caribbean, 75 percent of malaria infections are caused by *P. vivax*[4,5] and are rarely fatal, while 25 percent are caused by the more lethal *P. falciparum*, the dominant malaria parasite in Africa. *P. vivax* malaria, thought not an intrinsically life threatening disease, has been widely recognized to be understudied and to merit increase research and public policy attention.[4,6–11] The global burden of *P. vivax* malaria has been under appreciated and yet the morbidity associated with this infection and its

spectrum of disease is largely neglected;[4,8,12] therefore, malaria in the Americas has been widely neglected. A total of 1.06 million new cases of malaria were reported in the Americas in 1997, and 247,229 (23.3%) of these cases were from Peru.[13] Since then, the number of malaria reported in the Americas decreased from 1,150,103 cases and 348 deaths in 2000 to 882,361 cases and 156 deaths in 2004, reflecting a 23% reduction in the number of cases in the region and a 55% decrease in the overall number of malaria-attributed deaths in that period. The case fatality rate due to *P. falciparum* in the region also decreased from 13 per 10,000 cases in 2000 to 7 per 10,000 in 2004.

In contrast, Peru has had the largest net increase of 26% in malaria cases and the second highest number of malaria cases in the Americas reported from 2000 to 2004.[13] More than 60% of malaria in Peru occurs in the peri-Iquitos area, the capital city of the Peruvian Amazon Region of Loreto (PARL), mostly along forest fringes and `new' colonization areas. Malaria transmission intensity in the PARL is typically low (entomological inoculation rate (EIR) < 1 infective bite per year), few *Anopheles* spp. vector mosquitoes are infected with malaria parasites,[14–17] and mixed infections with *P. falciparum* and *P. vivax* are uncommon. Despite the low EIR and low transmission intensity in the Amazon region, asymptomatic malaria parasitemia is common.[17–20] Malaria transmission in the PARL is seasonal with an epidemic peak from February to July and similar to other malaria-

endemic countries, control measures are based on mainly passive surveillance with sporadic active surveillance campaigns. These measures are incorporated into general local health services within a partially decentralized National Malaria Program (NMP) of the Ministry of Health (MoH). During the 1980s and 1990s dramatic changes occurred in malaria transmission (Fig. 2.1), especially because of optimal environmental conditions for proliferation and spread of malaria vectors (i.e. deforestation), emergence of chloroquine-resistant *P. falciparum*, marked incidence of *P. vivax* malaria, entry of *Anopheles darlingii* into the ecology of the PARL mosquito population,[15,16] presence of asymptomatic persistent reservoirs for continuous reinfection[12] and ability of the primary parasite *P. vivax* to cause relapsing cases.

In addition to the well-recognized contributions of deforestation,[14,23] human sociodemographic factors and behaviors are likely to be important contributors to the reemergence and local reintroduction of malaria as a public health problem throughout the Amazon region. A major livelihood of people living in the rural areas surrounding Iquitos city is based on agriculture, fishing, logging and harvesting wood for charcoal at sites away from permanent domiciles. People with such work may camp outside for weeks or months, later returning home. These activities directly place people at risk for *Anopheles* mosquito bites, which in the PARL is primarily *An. darlingi*,[6,7,15,16] and bring people with malaria parasitemia from diverse places together. Collectively, these considerations drive the hypothesis that

people often acquire malaria outside their home villages and drive the dispersion of new parasite strains. Taking into account these socio-demographic considerations, we hypothesize that regional malaria control policies must consider how human behavior leads to the movement of malaria parasites. People with such epidemiological characteristics would be prime targets among who to deploy malaria insecticide treated bednets, chemoprophylaxis and/or transmission blocking strategies.

Antimalarial drugs are given free of cost by the Peruvian National Malaria Program (NMP) under a Directly Observed Therapy (DOT) protocol but in many geographically far, hard-to-access villages, enforcement of this policy may be less rigid than in villages closer to Iquitos. In remote places where these human reservoirs of malaria transmission may be frequently traveling, malaria diagnosis is not available. Despite the morbidity and large numbers of malaria cases occurring each year in the PARL, the region is considered hypoendemic (low malaria transmission).[17–19,24–26] A comprehensive description of malaria burden and risk factors in Peru, a malaria-hypoendemic region with relatively good access to diagnosis, has not been previously reviewed. Therefore, the present study attempted to describe the occurrence and identify risk factors related to malaria in villages of the PARL, to guide researchers and decision-makers in targeting intervention efforts and to provide additional insight into malaria transmission patterns in the study area.

MATERIALS AND METHODS

Study sites. A retrospective cohort study was carried out in 13 villages of Maynas, province of the PARL in the northeastern region of Peru, all located in one of the main axes of malaria transmission (Fig. 2.2). Loreto has a population of 964,195 inhabitants and Iquitos is a heavily populated metropolitan area (~60% of Loreto's population), located 120 meters above sea level at latitude 3.44°S and longitude 73.15°W on the bank of the Amazon River. The climate is tropical, with an average temperature of 27.5°C and a mean annual precipitation exceeding 2,500 mm. Monthly rainfalls are greatest in March (310 mm) and lowest in August (150 mm). Iquitos is relatively isolated, accessed only by air or by river and the majority of the population is of mixed Spanish and American Indian ancestry. Iquitos is also a tourist center, and many European and North American visitors pass through the city and surrounding areas on ecotourism expeditions. There are many small villages located on the banks of the Amazon River and its tributaries, within few hours by boat from Iquitos. People living in these villages are mainly involved in subsistence agriculture, fishing, harvesting wood for charcoal, and logging activities. In each village, composed of several hundreds to a few thousands of people, houses are modest, made of simple wood or concrete block construction with palm thatch or corrugated aluminum roofs, and usually lack running water, and electricity (Fig. 2.3). Considering the region's geographical isolation from the rest of Peru, health services to people living within the surrounding areas of Iquitos are relatively good and accessible.

Four health establishments provide health services to the study villages, described as follows:

Santo Tomas Health Post (STO)—Located 16 km far from Iquitos by road and surrounded by the Nanay River (Fig. 2.3) it is a referral health center for 3 villages: La Union (147 houses), 13 de Diciembre (35 houses), and Santo Tomas (302 houses). The whole population accounted for approximately 2,650 people.

San Jose de Lupuna Health Post (SJL)—Located 10 km from Iquitos, accessible through Nanay River, is referral for 4 villages: San Pedro (51 houses), Santa Rita (79 houses) (Fig. 2.2), Fray Martin (34 houses), and San Jose de Lupuna (84 houses), with a whole population of approximately 1,250 people.

Padrecocha Health Post (PAD)—Located 6 km from Iquitos, accessible only through the Nanay River, it is a referral health post for 3 villages: San Andres (28 houses), Nueva Vida (7 houses), and Padrecocha (324 houses), accounting for a population of approximately 1,800.

Mazan Health Center (MAZ)—Located 50 km northeast of Iquitos (40 minutes by speedboat), it is surrounded by the Amazon River to the south and the Napo River to the north, strategically near the confluence of the Napo and Amazon rivers. There is a small road of 3.4 km that crosses the portion of land between the 2 rivers and connects both rivers. Its geographic situation allowed Mazan to mediate commerce between all villages located in margin of the Napo River and Iquitos city through a fluvial wharf designed to attend boats of

up to 60 tons of movement and lifting capacity of up to 50,000 tons to the year. Three villages surrounding the health center were part of the study: San Jose, Puerto Alegre, and Mazan, totalizing around 4,200 people.

There is one malaria diagnostic laboratory (microscopy) in each of these four health establishments, responsible for providing diagnostic services and complete treatment against malaria 6 days a week. Malaria diagnosis in these areas follows standard procedures as defined by the MoH in Peru. Standard malaria therapies are: chloroquine for 3 days (10mg/ kg on days 1 and 2, and 5mg/kg on day 3), plus primaquine for 7 days (0.5 mg/kg/day) for *P. vivax* malaria; mefloquine (12.5 mg/kg/day for 2 days) plus artesunate (4 mg/kg/day for 3 days) for *P. falciparum* malaria.

Study population. It was composed by individuals of all ages living in the study sites; if a mother was not available, only the father would answer the questionnaire on behalf of children if they were too young to do so. The overall population comprised roughly 9,700 inhabitants, and 9,195 (95%) subjects participated in the study. Inhabitants who did not participate in the study were due to not finding an adult in their house to answer the questionnaire.

Data collection procedures. Ten health promoters from the study villages, 2 laboratory technicians and 1 experienced field nurse applying questionnaires were trained on the purpose of each item of the study questionnaire. Individual and household interviews recorded information on characteristics such as demographics, socioeconomic status, occupation, travel history and lifetime exposure to malaria. In the Mazan area questionnaires were applied during

May-June 2006; in the other areas during December-February 2007. Households were visited up to 3 times to find an adult to answer the questionnaire. The assembly of the cohort, and follow-up to determine the outcome variable (lifetime malaria episodes), has all happened in the past and was obtained by reviewing malaria program registries in the health establishments of the study areas; then it was complemented by self-reported lifetime malaria episodes at the time of questionnaire.

Laboratory procedures. To estimate malaria prevalence we performed examinations which routinely are thick blood smears; briefly, in the field 2 thick blood smears were taken, air-dried and stained with giemsa. Well-trained lab technicians examined 100 high-power fields of the thick blood smear for identification of malaria parasites. Treatment was provided for every subject with a positive smear and according to the Peruvian MoH.

Definition of malaria outcome. The outcome was `*Lifetime malaria episodes*', both types of malaria in the region (*P. falciparum* and *P. vivax*) were used, and it was categorized as: never, some (1 to 3 episodes), or several (≥ 4 episodes).

Potential risk factors. Variables selected explored as indicators of exposure to malaria in this study were: gender, health post, age, education, job, travel history and household variables. Health Posts were MAZ, STO, SJL, and PAD. *Age* was categorized as <5, [5–15>, [15–45>, ≥45 years old because it nicely divided the data into 4 groups and also treated as a continuous variable with increments of 10 years for more meaningful results. *Education* was

categorized as Technical-Superior, Secondary, Primary, Illiterate, and Under school age. *Job* was categorized as Commerce-taxi, Logger, Fishing, Agriculture, Housewife, Student, Unemployed, Woodcutter for charcoal, Urban Redevelopment Program building), and Others. Whether travel to rural areas outside of the home village was categorized as a yes-no question: jobs considered as requiring going outside village were Logging, Fishing, Agriculture, and Woodcutter for charcoal. *Travel history* was categorized as a yes/no question regarding if the subject traveled or not, travel to Iquitos was excluded because transmission is less likely to occur in this city. Three definitions of travel were used, `T1' was limited to the month previous to the census and of at least 3 days-duration and ≥ 10km as determined by location of place visited using determined by river used to travel, type of transportation, near village of place visited and duration of trip. `T2' referred to month previous to census but considered any distance & duration. `T3' referred to any of the last 4 or less malaria episodes for ≥ 10km and staying out of village at least for 3 days; for subjects with never malaria, T3 referred to lifetime travel of such characteristics. *Living with a logger or agriculturist* was categorized as yes for people who live in the same house with at least one logger or agriculturists. *Household variables* such as exterior / interior wall materials (brick, cement, wood calamine, etc), location of kitchen (exterior, interior), roof material (calamine, palms), rate of bednets per beds, possessions (radio, television, etc). All household variables were not significantly associated with outcome; therefore, tables are not shown for these variables.

Data analysis and statistical procedures. Data were analyzed using SAS v.9.1. Since our outcome is not just dichotomous but consists of 3 categories, then polychotomous logistic regression was used for analysis.[21] In particular, this tool allowed us to assess effects on odds of several vs. never and some vs. never malaria episodes lifetime. First, we performed polychotomous logistic regression for each variable independently as univariate analysis; second, we performed polychotomous logistic regression for all variables together as a multinomial analysis. The significance level for all hypothesis testing was set at $p \leq 0.05$ and confidence intervals accordingly.

Ethical considerations. This study was approved by the Ethical Committees of Universidad Peruana Cayetano Heredia and Asociación Benéfica Prisma in Lima, Peru, by the Peruvian Amazon Region Directorate of Health and the Institutional Review Boards of the University of California San Diego and the Johns Hopkins Bloomberg School of Public Health.

RESULTS

Description of study population. The study population consisted of 9,195 individuals, 14%, 17%, 29%, and 40% were from SJL, PAD, STO, and MAZ, respectively (Table 2.1). A total of 53% were male and 13% younger than 5 years old, with a mean age of 24.4 years, and ranging from <1 to 92 years. The median monthly family income (S/.483 New Soles or $146 U.S. dollars) was under the Peruvian minimum, which was S/.550 New Soles ($167 U.S. dollars) in 2007 (Table 2.1). A homogeneous pattern of behaviors regarding health care service use was observed in the study population. All individuals who reported having had at least one malaria episode referred had used the MoH laboratories for malaria diagnosis and treatment. Approximately one third of the population (33.9%) reported not having experienced at least one episode of malaria during his (her) lifetime. Proportion of individuals reporting never *P. falciparum* (68.6%) was almost twice the proportion of individuals reporting never *P. vivax* (38.5%). The median number of malaria episodes during lifetime was 3.0. The study population reported a mean time living in malaria endemic areas of approximately 17 years with a median of 12 years, and 32% had lived in these areas for less than 5 years. Use of individual protective measures against mosquito bites was rarely reported, except for regular work clothes.

Considering people ≥ 18 years old (5,033 or 55% of the population): The majority had attended school a mean of 7.1 years and 4.8% never went to school; 43.3%, 45.4% and 6.5% attended primary school, secondary school

and technical or professional studies, respectively. Considering people < 18 years old, 32.4% of individuals were under age to go to school (< 6 years old) and 67.6% were on age to go to school from which 2.1% never went to school. The seven most reported past occupations were housewife (31.2%), agriculture (13.5%), commerce (8.2%), logging (7.5%), fishing (4.7%), woodcutter/coal (2.4%), mototaxi (2.2%) and crafts (3.4%). 27.3% referred routinely going outside village for job purposes. People with never malaria was equally represented by children < 5 years old (29.6%), 5–15 years old (33.7%), ≥ 15 years old (36.7%). The group of several malaria episodes lifetime was composed mainly of 15–45 years old subjects (57%), 56.2% males and 43.8% females.

The prevalence of malaria during the study period is shown in Table 2.2. Among 5556 blood smears examined, 205 cases of malaria parasitemia were identified: 172 of *P.* vivax, 33 of *P. falciparum* and none mixed infections. Overall, malaria prevalence during the study visit was 3.7% and a ratio of *P. falciparum* to *P. vivax* of 1:0.19. Most (66.13%) of the study population had been previously exposed to malaria with 86.4%, 85.2%, 76.4% and 50.3% for each health establishments PAD, SJL, STO, and MAZ, respectively. The annual parasite index (API) was calculated from the number of parsitologically confirmed cases of malaria yearly from 2002 to 2007 (Table 2.3) as it relates to the average yearly population in the villages during the year. These high rates are comparable with hyperendemic areas where there are multiple annual malaria episodes per child, plus occasional cases among adults. In the

study villages as in most of the Peruvian Amazon Region the distribution of malaria cases is entirely different of that observed in African and Pacific Hyperendemic areas of malaria. On average from 2002 to 2007, in the health establishments' study villages the majority of the cases occur in adults (≥ 15 years old): San Jose de Lupuna (52.3%), Santo Tomas (69.2%), Padrecocha (66.2%), and Mazan (70.3%). This is the same for most of the health establishments in Loreto where overall we had 57.9%, 52.8%, 57% and 59.4% of the malaria cases in adults from 2002 to 2007, respectively. More than 80% of the cases from 2002 to 2007 were reported during the first 25–35 epidemiologic weeks yearly (Table 2.3).

Risk factors for malaria lifetime–Univariate/Multivariate analysis. The total number of lifetime malaria episodes was found to be positively associated with age (categorized and continuous), gender, health establishment, job, travel history (T1, T2 and T3), job outside village, education, and living with a logger or agriculturist, based on univariate analysis (Table 2.4). Considering that there were three types of travel history and to avoid colinearity issues, we selected T3 for travel history for multivariate modeling using polychotomous logistic regression (Table 2.5). Variables in the multivariate analysis included the following: age, gender, education, job, health establishment, travel history (T3), and living with a logger or agriculturist. After adjustment, age, health establishment, type of job, travel history and living with a logger or agriculturist remained significantly associated with the number of lifetime malaria episodes;

gender was borderline significant for some vs. never malaria but not significant for several vs. never malaria, and education was not significant.

Age—The odds of some malaria episodes (relative to never) increase by a factor of 1.2 for every 10 years increase in age after adjusting for gender, education, health establishment, job type, travel history, and living with a logger or agriculturist; with a 95%CI from 1.2 to 1.3. The odds of several malaria episodes (relative to never) increase by a factor of 1.6 for every 10 years increase in age after adjusting for gender, education, health establishment, job type, and travel history; with a 95%CI from 1.5 to 1.7. The odds of some malaria episodes (relative to never) are 2.9 times higher for 15 or older years old subjects than for less than 15 years old subjects after adjusting for gender, education, health establishment, job type, travel history, and living with a logger or agriculturist; with a 95%CI from 1.2 to 2.0. The odds of several malaria episodes (relative to never) are 3.0 times higher for 15 or older subjects than for subjects less than 15 years old after adjusting for gender, education, health establishment, job type, travel history, and living with a logger or agriculturist; with a 95% CI from 2.2 to 4.2.

Gender—The odds of some malaria episodes (relative to never) are 1.2 times higher for male subjects than for female subjects after adjusting for age, education, health establishment, job type, travel history, and living with a logger or agriculturist; with a 95%CI from 1.0 to 1.4. The association between several malaria episodes (relative to never) and gender was not significant after adjustment.

Health establishment—From univariate analysis we chose Mazan as the reference category. *Padrecocha:* The odds of some malaria episodes (relative to never) were 10.1 times higher for subjects living in Padrecocha than for subjects living in Mazan after adjusting for age, gender, education, job type, travel history, and living with a logger or agriculturist; with a 95%CI from 7.2 to 14.2. The odds of several malaria episodes (relative to never) are 40.6 times higher for subjects living in Padrecocha than for subjects living in Mazan after adjusting for age, gender, education, job type, travel history, and living with a logger or agriculturist; with a 95%CI from 27.6 to 59.6. *San Jose de Lupuna:* The odds of some malaria episodes (relative to never) were 12.8 times higher for subjects living in San Jose de Lupuna than for subjects living in Mazan after adjusting for age, gender, education, job type, travel history, and living with a logger or agriculturist; with a 95%CI from 10.2 to 16.2. The odds of several malaria episodes (relative to never) were 127.1 times higher for subjects living in San Jose de Lupuna than for subjects living in Mazan after adjusting for age, gender, education, job type, travel history, and living with a logger or agriculturist; with a 95%CI from 97.8 to 168.8. *Santo Tomas:* The odds of some malaria episodes (relative to never) were 8.6 times higher for subjects living in Santo Tomas than for subjects living in Mazan after adjusting for age, gender, education, job type, travel history, and living with a logger or agriculturist; with a 95% CI from 7.3 to 10.2. The odds of several malaria episodes (relative to never) were 39.2 times higher for subjects living in Santo Tomas than for subjects living in Mazan after adjusting for age,

gender, education, job type, travel history, and living with a logger or agriculturist; with a 95% CI from 31 to 49.

Occupation—People working in commerce or mototaxi (are inside village boundaries in the market or small grocery stores, not at home, driving on the roads of these villages or going to Iquitos) were used together as a reference category. *Agriculture:* The odds of some malaria episodes (relative to never) were 3.2 times higher for subjects working on agriculture than for subjects working on commerce/mototaxi after adjusting for age, gender, education, travel history, and living with a logger or agriculturist; with a 95% CI from 2.0 to 5.2. The odds of several malaria episodes (relative to never) were 4.5 times higher for subjects working on agriculture than for subjects working on commerce/mototaxi after adjusting for age, gender, education, travel history, and living with a logger or agriculturist; with a 95% CI from 2.7 to 7.4. *Housewife:* The odds of some malaria episodes (relative to never) were 1.7 times higher for housewives than for subjects working on commerce/mototaxi after adjusting for age, gender, education, travel history, and living with a logger or agriculturist; with a 95%CI from 1.3 to 2.4. The odds of several malaria episodes (relative to never) were 1.7 times higher for housewives than for subjects working on commerce/mototaxi after adjusting for age, gender, education, travel history, and living with a logger or agriculturist; with a 95% CI from 1.2 to 2.3. *Logging.* The odds of some malaria episodes (relative to never) were 2.6 times higher for subjects working on logging than for subjects working on commerce/mototaxi after adjusting for age, gender, education,

travel history, and living with a logger or agriculturist; with a 95% CI from 1.5 to 4.6. The odds of several malaria episodes (relative to never) were 5.0 times higher for subjects working on logging than for subjects working on commerce/mototaxi after adjusting for age, gender, education, travel history, and living with a logger or agriculturist; with a 95%CI from 2.8 to 9.0.

Travel history (T3)—Iquitos as a destination was not considered as traveling regardless of time. In the multivariate analysis we used only T3 for travel history: it referred to the month before one of the last four malaria episodes; and for subjects with never malaria, it referred to lifetime travel to further than 10 km and/or for at least 3 days. The odds of some malaria episodes (relative to never) were 31.1 times higher for subjects who traveled than for subjects who did not travel (as defined by T3) after adjusting for age, gender, education, health establishment, job type, and living with a logger or agriculturist; with a 95% CI from 24.7 to 39.2. The odds of several malaria episodes (relative to never) were 57.8 times higher for subjects who traveled than for subjects who did not travel (as defined by T3) after adjusting for age, gender, education, health establishment, job type, and living with a logger or agriculturist, with a 95%CI from 49.9 to 76.1.

Living with a person working on logging or agriculture—The odds of some malaria episodes (relative to never) were 1.8 times higher for subjects who lived with a logger or agriculturist than for subjects who did not live with a person working on such activities, after adjusting for age, gender, education, health establishment, job type, and travel history; with a 95%CI from 1.6 to

2.1. The odds of several malaria episodes (relative to never) were 2.2 times higher for subjects who lived with a logger or agriculturist than for a subject who did not live with a person working on such activities, after adjusting for age, gender, education, health establishment, job type, and travel history; with a 95%CI from 1.9 to 2.6.

DISCUSSION

From 1880 to 1945, the Amazon was the epicenter of-the `rubber boom', an important period in the socio-economic history of countries with Amazon territories such as Peru, Brazil, Colombia and Ecuador, triggering the migration process, wealth and socio-cultural transformations. During that period all study areas were established with internal migration and colonization with malaria-naive people from the Peruvian Andes. In 1954, an eradication campaign was launched in the Americas which in 1955 became worldwide, and in Peru the National Service for Malaria Eradication (NSME) was formed. In 1970 with the entrance of a military government which believed that the solution to rural poverty was not in the development of public health but in agrarian reform, the NSME was eliminated and the malaria control budget fell drastically. Other political factors that contributed to the reemergence of malaria were the discontinuation of the U.S. financial help for malaria eradication worldwide.

Few studies have focused on associated risk factors for malaria in communities originating from frontier settlements in the Amazon Basin of Peru. An insightful analysis of frontier malaria in Amazonia comprises three stages: epidemic, transition and endemic.[27] The first stage is characterized by high malaria rates and the inefficacy of traditional control measures, whereas the transition period is marked by declines in malaria rates that are partially explained by improved health infrastructure and reduced mobility of settlers. Malaria transmission becomes less intense and more stable in the final

stages. Our study provides an example of how heterogeneous is the pattern of malaria due to different individual and group sociodemographic and behavioral characteristics.

Historically, failure to consider population movement contributed to failure of malaria eradication campaigns in the 1950s and 1960s.[28,29] As people move, they can increase their risk for acquiring the disease through the ways in which they change the environment.[30,31] Such activities can create more favorable habitats for *Anopheles* mosquitoes; at the same time, workers may have increase exposure to the vector. When economic or other needs can no longer be met in a particular environment, people move elsewhere. The `push factor' could be environmental degradation, population pressure on land, employment, etc. When people believe that a move elsewhere will provide new and attractive opportunities, a 'pull factor' is involved such as better political, economical or social opportunities.[32,33]

The Peruvian Department of Loreto remains an important migration destination for people from other cities in Peru (internal migration) and from frontier cities in Colombia and Brazil (external migration). These migration patterns facilitate the movement both of malaria immunes and non-immunes among areas with varying levels of vector density, resulting in a potentially explosive combination for high transmission rates and spread of infection over a wide area such as occurred in Amazonia in the 1990s.[6,7] In terms of spatial dimensions, such human population movements to and from malarious areas

are of epidemiologic importance of particular importance in both sustaining and reintroducing malaria. People at high risk for malaria including asymptomatic parasitemia due to acquired clinical immunity over time (e.g. loggers, agriculturists, etc) behave as active transmitters harboring the parasite and transmitting the disease when they move back to areas of low or sporadic transmission, therefore they are likely to be important sources of seasonal epidemics in the region. Since occupational activities are seasonal the same people behave as passive acquirers when exposed to the disease through movement to higher transmission intensity environments; family members and others with low-level or no immunity are often epidemiologically linked to those clinically immune, which increases the risk for disease spread.[28]

Another sociodemographic feature observed in our study sites is a variable degree of circulation for economic purposes, that is, going between a fixed residence and a site of field labor; such circulation encompasses a variety of movements, usually short-term and cyclical involving no longstanding change of residence.[33] Daily circulation involves leaving a place of residence for up to 24 hours, periodic circulation may vary from 1 night to 1 year, and seasonal circulation is defined by marked seasonality in the physical or economic environment. The study areas exemplify clearly all types of circulation for different activities such as cultivation, logging, fishing, woodcutters for charcoal and tourism. The settlers in the study areas as in most villages in the PARL are highly mobile, moving with daily, periodic and

seasonal circulation, from settlements in unstable disease endemic regions to hyperendemic-disease regions of the rainforests. This mobility keeps settlements unstable and at high risk for epidemics through the constant flow of parasitemic laborers.[34,35]

Nearly 20% of the Amazon rainforest has disappeared since 1970, which presents a major threat to the world's carbon and climate balance. There is a substantial forest disturbance adjacent to areas set aside for legal logging operations, another important ecological and environmental concern.[36] Moreover, between 1999 and 2005, 75% of the rain forest damage was found within 20 km of the roads. However, even within those limits, forests set aside by the government were more than 4 times better protected than areas not designated for conservation.[27,36] The Loreto Regional Government is about to build a highway from the frontier river village of Mazan, through the Amazon jungle, to the city of Iquitos. This new road will serve as a bidirectional movement of groups of people identified as developing several malaria episodes lifetime in our study; therefore, we have the opportunity to anticipate, and to study: 1) a potentially huge source of reintroduction of malaria parasites and vectors to areas of Iquitos where there is very low or no transmission but the conditions are most likely favorable and 2) the increasing entrance of non-immune subjects looking for better opportunities.

The analysis of occupational activities allowed us to identify two major groups: 1) subjects with mainly domestic activity, including housewives, students and unemployed adults; and 2) subjects working in agriculture, crafts, harvesting wood for charcoal, logging and fishing (Table 2.4). When performing the multivariate analysis, agriculturists, housewives, loggers and students remained statistically significant associated with several malaria episodes lifetime (Table 2.5). The latter analysis defines the main risk group among subjects working in the rain forest or in agricultural activities and its partners at home. Alongside the Amazon River and its many tributaries, poverty-stricken loggers and agriculturists move deep into the rainforest, in areas where malaria is prevalent, without taking any precaution and for meager wages, making them the mosquitoes' main victims. Inside villages people who live with a person working in such activities may secondarily acquire malaria.

At least two findings have clear implications for designing appropriate interventions against malaria in our site and other settings with similar endemicity. First, malaria morbidity is clearly associated not only with forest related activities such as logging but also with agriculture activities, sometimes acquired in areas away from villages. Such activities together represent the primary occupation of 21.3% study participants. Because land clearing also leads to changes in relative vector abundance that may favor malaria transmission in such a modified environment, this activity plays a crucial role in maintaining malaria transmission. These circumstances are reminiscent of the

well-studied forest-malaria phenomenon in Southeast Asia and call for specific individual and community level interventions to reduce the risk of forest-related malaria in frontier settlements of the Amazon Basin. Second, the probability of having malaria clearly increased with previous history of travel which is of tremendous importance and give us the opportunity for planning and developing a malaria screening site at each point of entrance in villages and regardless of legal issues, health authorities need to coordinate malaria control interventions with logging companies to avoid incomplete treatments without prescription and to deploy basic diagnosis units and surveillance for malaria through the areas of such activities.

Drug treatment kills the asexual stages of *P. falciparum* before they are able to produce gametocytes so appropriate deployment of health services and drug treatment of *P. falciparum* infections can be very effective (until drug-resistance develops). *P. vivax* is less vulnerable to control by these means because mosquitoes will mostly have become infected during a pre-symptomatic period and therefore before drug treatment could have prevented onward transmission of the parasites through mosquitoes.[6] On the other hand, vector control has historically been the only effective means of malaria control but anti-mosquito abatement programs are difficult to maintain politically and ecologically on a long-term basis,[32] particularly because *Anopheles darlingi* is essentially sylvatic and bites not only in a crepuscular (sunset) mode but at various times between dusk and dawn in the Loreto region of Peru (Jan Conn and Marta Moreno unpublished). There is evidence

that a substantial proportion of people 5 years or older in villages around Iquitos have asymptomatic malaria parasitemia, whether *P. vivax* or *P. falciparum*, which is likely the dominant reservoir of continued transmission.[17] We have observed (Vinetz and Gilman, unpublished) that in villages near Iquitos, approximately 4–8% of all people sampled through a cross-sectional active surveillance had malaria parasitemia as determined by light microscopy. Of these, ~2/3 had no symptoms referable to malaria within the prior 2 weeks. These data are mirrored by findings in Brazilian Amazonia[19,26] and we and others have commented on the observation that asymptomatic reservoirs of malaria parasitemia are likely to be reservoirs of continuing malaria transmission.[37]

We have identified target populations for testing and deploying a malaria transmission- blocking vaccine (TBV) to prevent malaria parasites from being transmitted from the only known reservoir, humans, to mosquitoes. Theoretically, TBVs would have the same effect on reducing malaria as eliminating the mosquito vector preventing mosquitoes from becoming infected with the parasite after ingestion of blood from an infected individual. TBVs would not prevent recipients from developing malaria, but the idea is to develop a type of herd immunity within human populations that serve as important reservoirs of transmission. Targeting the people who serve as the most important reservoirs for mosquito infection with a TBV would have the greatest effect on malaria transmission within a population.[38,39] Alternative

population-based strategies other than TBVs to prevent malaria transmission could also include radical treatment or malaria chemoprophylaxis of risk groups as described herein, but would be practically difficult to implement.

TBVs are designed to induce an antibody response in humans that will block the parasite's infectivity to mosquitoes, hence spread of parasites between people and reduce the potential for infected returning travelers, migrants and circulators to introduce or re-introduce malaria into a given area. The approach is as follows: 1) Humans are vaccinated with recombinant mosquito/sexual-stage specific protein(s). 2) When a mosquito ingests a blood meal, vaccine-induced antibodies are taken up along with parasites (gametocytes). 3) Ingested antibodies would then interact with parasite surface or secreted proteins within the mosquito midgut, preventing the parasite from infecting the mosquito. If enough people within a population develop such antibodies, malaria transmission can be reduced, possibly even eliminated.[39] Additionally, TBVs would reduce the spread of drug- or vaccine-selected mutant parasites by reducing genetic recombination of the parasites within the mosquito. The mosquito stage of malaria parasites is a genetic bottleneck: of the tens or hundreds of gametocytes that enter the mosquito, derived from millions to hundreds of millions of erythrocytic parasites, only a few oocysts (~$10^0 - 10^1$) develop within the mosquito midgut.[40–42] An effective TBV may extend the useful life of newly developed drugs or other malaria vaccines by reducing the passage of mutant parasites from drug- or vaccine-treated humans to mosquitoes.

This study provides new insights into mechanisms of persistence, introductions, and reemergence of malaria. The considerations discussed there towards new approaches to control malaria based on the considerations of sociodemographic and behavioral factors in the hypoendemic Peruvian Amazon settings. The generalizability of these observations to more intensive transmission settings needs to be determined. This information may help guide decision makers to specify effective control measures targeted to this population and transmission situation. Moreover, it provides measurable evidence of the burden of malaria in this area, especially regarding type of occupation and travel history definitions, which may influence political commitment with regard to this relevant public health problem. Furthermore, this epidemiologic and sociodemographic information is essential for establishing field sites for testing malaria TBVs, which by necessity will need to be deployed on a micro-environmental scale. Population movements that either place people at risk for malaria or cause them to pose a risk to others cannot be stopped. However, prevention measures can address the causes of these movements which, in developing countries, are often prompted by need rather than choice. These data support our hypothesis that persons infected with malaria are likely to have contracted it outside the limits of the village. Migratory and circulatory labors appear to be major determinants of human reservoirs of *P. vivax*. Malaria control in such areas could be achieved most efficiently by the deployment of a targeted intervention in high risk groups using a TBV.

Further studies are needed to measure the magnitude of *P. vivax* relapses occurring in the Peruvian Amazon to allow us quantify the burden of this phenomena which is known to cause a cumulative experience of 10–30 episodes of malaria during lifetime.[6] Further analysis of data generated by this ongoing cohort study is expected to provide additional insights into the phenomenon of frontier malaria in the Amazon Basin.

LIMITATIONS

Potentially, the most important source of bias in measuring number of infections lifetime was recall bias. We are not concerned about recall bias because of two reasons: 1) as long as memory failure is equal amongst individuals, recall bias will not occur, and 2) we treated outcome as categorical which we definitively think avoided recall bias.

ACKNOWLEDGMENTS

I thank the health promoters in Santo Tomas, La Union, Santa Clara, San Jose de Lupuna, San Pedro, Santa Rita, Fray Martin, Mazan, Puerto Alegre and San Jose along with their community leaders for their invaluable cooperation. We also thank Ministry of Health authorities at the Loreto Directorate of Health for their assistance.

The guidance and inspiration from Drs. Stephanie Brodine, John Weeks and Richard Garfein, members of Dr. Chuquiyauri's thesis committee in the San Diego State University-University of California San Diego Doctoral Program in Global Health (supported by NIH grant R25TW007500), is gratefully acknowledged.

I would like to acknowledge co-authors Maribel Paredes, Pablo Penataro, Sonia Torres, Silvia Marin, Alexander Tenorio, Kimberly Brouwer, Shira Abeles, Alejandro Llanos, Robert Gilman, and Margaret Kosek, for their contribution to this paper and permission to use it as part of my dissertation.

This work was been supported by U.S. Public Health Service grants D43TW007120, 1R01AI067727, U19AI089681 K24AI068903, and R01R0145999.

ABBREVIATIONS

PARL	Peruvian Amazon Region of Loreto
EIR	Entomological inoculation rate
NMP	National Malaria Program
MoH	Ministry of Health
STO	Santo Tomas Health Post
SJL	San Jose de Lupuna Health Post
PAD	Padrecocha Health Post
MAZ	Mazan Health Center
API	Annual parasite index
NSME	National Service for Malaria Eradication
TBV	Transmission-blocking vaccine

REFERENCES

1. Alonso PL, Brown G, Arevalo-Herrera M, Binka F, Chitnis C, Collins F, Doumbo OK, Greenwood B, Hall BF, Levine MM, Mendis K, Newman RD, Plowe CV, Rodriguez MH, Sinden R, Slutsker L, Tanner M. A research agenda to underpin malaria eradication. *PLoS Med*. 2011; 8:e1000406.

2. A research agenda for malaria eradication: cross-cutting issues for eradication. *PLoS Med*. 2011; 8:e1000404.

3. A Research Agenda for Malaria Eradication: Monitoring, Evaluation, and Surveillance. *PLoS Med*. 2011; 8:e1000400.

4. Price RN, Tjitra E, Guerra CA, Yeung S, White NJ, Anstey NM. Vivax malaria: neglected and not benign. *Am J Trop Med Hyg*. 2007; 77:79–87.

5. Duarte EC, Gyorkos TW, Pang L, Abrahamowicz M. Epidemiology of malaria in a hypoendemic Brazilian Amazon migrant population: a cohort study. *Am J Trop Med Hyg*. 2004; 70:229–37.

6. Aramburu Guarda J, Ramal Asayag C, Witzig R. Malaria reemergence in the Peruvian Amazon region. *Emerg Infect Dis*. 1999; 5:209–15.

7. Roper MH, Carrion Torres RS, Cava Goicochea CG, Andersen EM, Aramburu Guarda JS, Calampa C, Hightower AW, Magill AJ. The epidemiology of malaria in an epidemic area of the Peruvian Amazon. *Am J Trop Med Hyg*. 2000; 62:247–56.

8. Mendis K, Sina BJ, Marchesini P, Carter R. The neglected burden of Plasmodium vivax malaria. *Am J Trop Med Hyg*. 2001; 64:97–106.

9. Baird JK. Neglect of Plasmodium vivax malaria. *Trends Parasitol*. 2007; 23:533–9.

10. Sina B. Focus on *Plasmodium vivax*. *Trends Parasitol*. 2002; 18:287–9.

11. Sina B. Focus on *Plasmodium vivax*. *Trends Parasitol*. 2004:2–4.

12. Guerra CA, Howes RE, Patil AP, Gething PW, Van Boeckel TP, Temperley WH, Kabaria CW, Tatem AJ, Manh BH, Elyazar IR, Baird JK, Snow RW, Hay SI. The international limits and population at risk of Plasmodium vivax transmission in 2009. *PLoS Negl Trop Dis*. 2010; 4:e774.

13. PAHO. Incidence of Diseases under Epidemiological Surveillance. Pan American Health Organization; Washington, DC: 2007. Health Situation in the Americas: Basic Indicators, 1995–1998.

14. Vittor AY, Pan W, Gilman RH, Tielsch J, Glass G, Shields T, Sanchez-Lozano W, Pinedo VV, Salas-Cobos E, Flores S, Patz JA. Linking deforestation to malaria in the Amazon: characterization of the breeding habitat of the principal malaria vector, Anopheles darlingi. *Am J Trop Med Hyg*. 2009; 81:5–12.

15. Schoeler GB, Flores-Mendoza C, Fernandez R, Davila JR, Zyzak M. Geographical distribution of *Anopheles darlingi* in the Amazon Basin region of Peru. *J Am Mosq Control Assoc*. 2003; 19:286–96.

16. Flores-Mendoza C, Fernandez R, Escobedo-Vargas KS, Vela-Perez Q, Schoeler GB. Natural *Plasmodium* infections in *Anopheles darlingi* and *Anopheles benarrochi* (Diptera: Culicidae) from eastern Peru. *J Med Entomol*. 2004; 41:489–94.

17. Roshanravan B, Kari E, Gilman RH, Cabrera L, Lee E, Metcalfe J, Calderon M, Lescano AG, Montenegro-James S, Calampa C, Vinetz JM. Endemic malaria in the Peruvian Amazon region of Iquitos. *Am J Trop Med Hyg*. 2003; 69:45–52.

18. Camargo EP, Alves F, Pereira da Silva LH. Symptomless *Plasmodium vivax* infections in native Amazonians. *Lancet*. 1999; 353:1415–6.

19. Alves FP, Durlacher RR, Menezes MJ, Krieger H, Silva LH, Camargo EP. High prevalence of asymptomatic *Plasmodium vivax* and *Plasmodium falciparum* infections in native Amazonian populations. *Am J Trop Med Hyg*. 2002; 66:641–8.

20. Ladeia-Andrade S, Ferreira MU, de Carvalho ME, Curado I, Coura JR. Age-dependent acquisition of protective immunity to malaria in riverine populations of the Amazon Basin of Brazil. *Am J Trop Med Hyg*. 2009; 80:452–9.

21. Vittor AY, Gilman RH, Tielsch J, Glass G, Shields T, Lozano WS, Pinedo-Cancino V, Patz JA. The effect of deforestation on the human-biting rate of Anopheles darlingi, the primary vector of Falciparum malaria in the Peruvian Amazon. *Am J Trop Med Hyg*. 2006; 74:3–11.

22. Jordan SJ, Oliveira AL, Hernandez JN, Oster RA, Chattopadhyay D, Branch OH, Rayner JC. Malaria immunoepidemiology in low transmission: Correlation of infecting genotype and immune response

to domains of Plasmodium falciparum Merozoite Surface Protein-3. *Infect Immun*. 2011

23. Sutton PL, Neyra V, Hernandez JN, Branch OH. Plasmodium falciparum and Plasmodium vivax infections in the Peruvian Amazon: propagation of complex, multiple allele-type infections without super-infection. *Am J Trop Med Hyg.* 2009; 81:950–60.

24. Camargo LMA, Noronha E, Villalobos Salcedo JM, Dutra AP, Krieger H, da Silva LHP, Camargo EP. The epidemiology of malaria in Rondonia (Western Amazon region, Brazil): study of a riverine population. *Acta Tropica*. 1999; 72:1–11.

25. Hosmer, DW.; Lemeshow, S. Applied logistic regression. 2nd edition. 2000. Wiley series in probability and statistics.

26. Begg CB, Gray R. Calculation of polychotomous logistic regression parameters using individualized regression. *Biometrika*. 1984; 71:11–18.

27. De Castro MC, Monte-Mor RL, Sawyer DO, Singer BH. Malaria risk on the Amazon frontier. *Proc Natl Acad Sci*. 2006; 103:2452–7.

28. Prothero RM. Disease and mobility: a neglected factor in epidemiology. *Int J Epidemiol*. 1977; 6:259–67.

29. Bruce-Chwatt LJ. Movements of populations in relation to communicable disease in Africa. *East Afr Med J*. 1968; 45:266–75.

30. Service MW. Agricultural development and arthropod-borne diseases: a review. *Rev Saude Publica*. 1991; 25:165–78.

31. Guthmann JP, Hall AJ, Jaffar S, Palacios A, Lines J, Llanos-Cuentas A. Environmental risk factors for clinical malaria: a case-control study in the Grau region of Peru. *Trans R Soc Trop Med Hyg*. 2001; 95:577–83.

32. Kosinski, LA.; Prothero, RM. The study of migration. In: Kosinski, LA.; Prothero, RM., editors. People on move: studies on internal migration. Methuen; London:1975.p.1-38.

33. Gould, WTS.; Prothero, RM. Space and time in African population mobility. In: Kosinski, LA.; Prothero, RM., editors. People on the move: studies on internal migration. Methuen; London: 1975. p. 39-49.

34. Camargo LM, Ferreira MU, Krieger H, De Camargo EP, Da Silva LP. Unstable hypoendemic malaria in Rondonia (western Amazon region, Brazil): epidemic outbreaks and work-associated incidence in an agro-industrial rural settlement. *Am J Trop Med Hyg*. 1994; 51:16–25.

35. McGreevy PB, Dietze R, Prata A, Hembree SC. Effects of immigration on the prevalence of malaria in rural areas of the Amazon basin of Brazil. *Mem Inst Oswaldo Cruz*. 1989; 84:485–91.

36. Oliveira PJ, Asner GP, Knapp DE, Almeyda A, Galvan-Gildemeister R, Keene S, Raybin RF, Smith RC. Land-use allocation protects the Peruvian Amazon. *Science*. 2007; 317:1233–6.

37. Vinetz JM, Gilman RH. Asymptomatic *Plasmodium* parasitemia and the ecology of malaria transmission. *Am J Trop Med Hyg*. 2002; 66:639–40.

38. Carter R. Spatial simulation of malaria transmission and its control by malaria transmission blocking vaccination. *Int J Parasitol*. 2002; 32:1617–24.

39. Carter R, Mendis KN, Roberts D. Spatial targeting of interventions against malaria. Bulletin of the WHO. 2000; 78:1401–11.

40. Do Rosario VE, Vaughan JA, Coleman RE. Analysis of the sporogonic development of Plasmodium falciparum and Plasmodium berghei in anopheline mosquitoes. *Parassitologia*. 1989; 31:101–11.

41. Billingsley PF, Medley GF, Charlwood D, Sinden RE. Relationship between prevalence and intensity of Plasmodium falciparum infection in natural populations of Anopheles mosquitoes. *Am J Trop Med Hyg*. 1994; 51:260–70.

42. Vaughan JA, Narum D, Azad AF. Plasmodium berghei ookinete densities in three anopheline species. *J Parasitol*. 1991; 77:758–61.

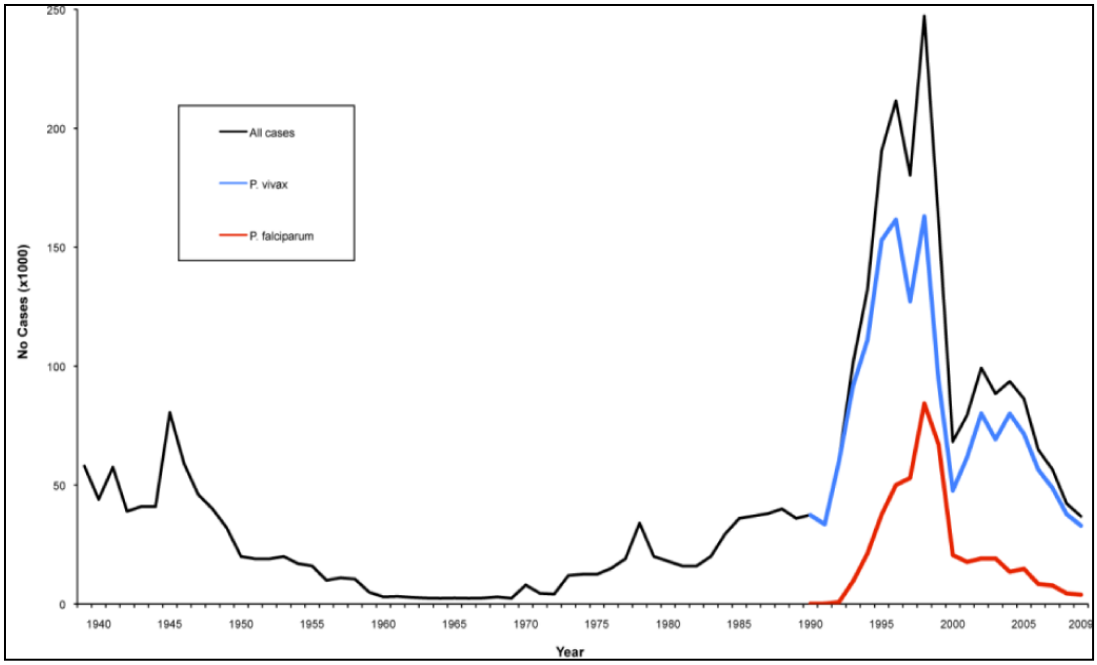

Figure 2.1. Malaria Cases in Peru: 1939–2010. Cases of malaria due to *P. falciparum* and *P. vivax* were not differentiated nor reported systematically until 1990. Source: Peruvian Ministry of Health, Lima, Peru.

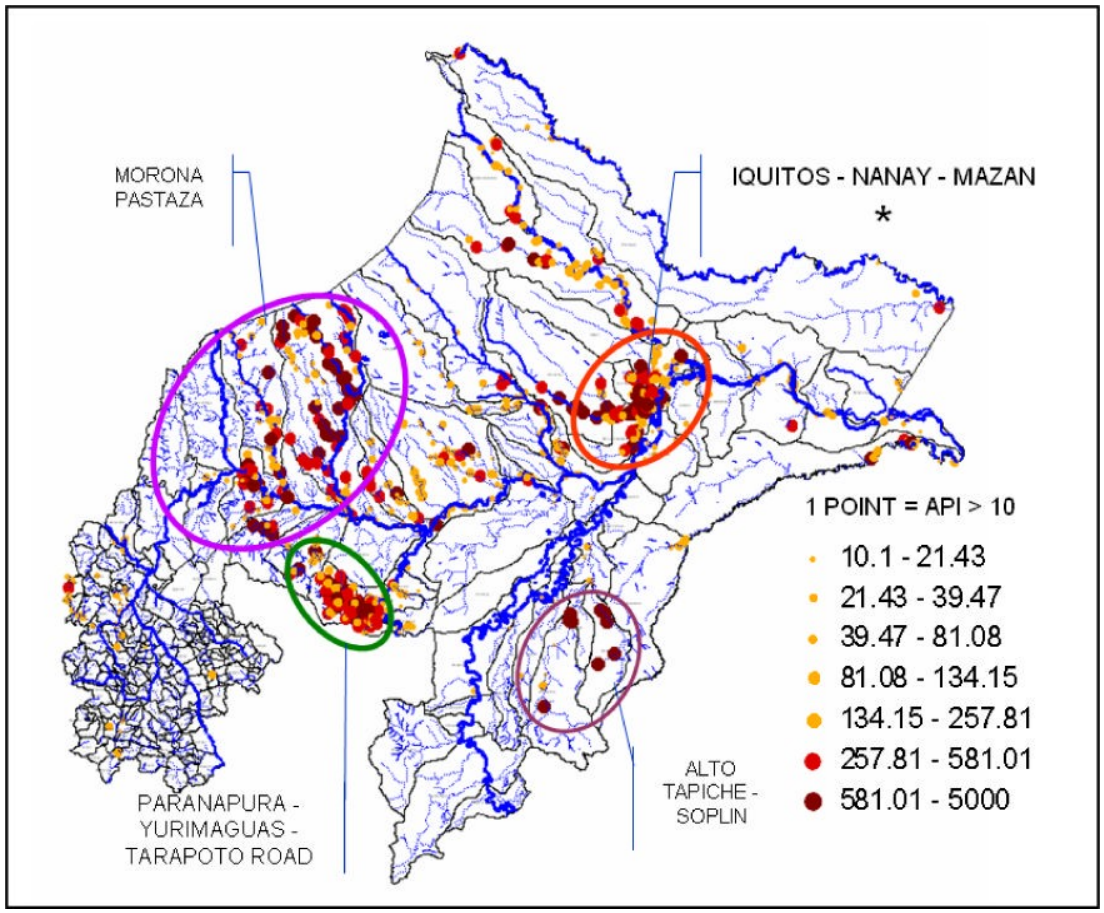

Figure 2.2. Axes of malaria transmission in Loreto, Peru-2007. Study sites location. Source: Loreto Regional Health Directorate, Iquitos, Loreto, Peru.

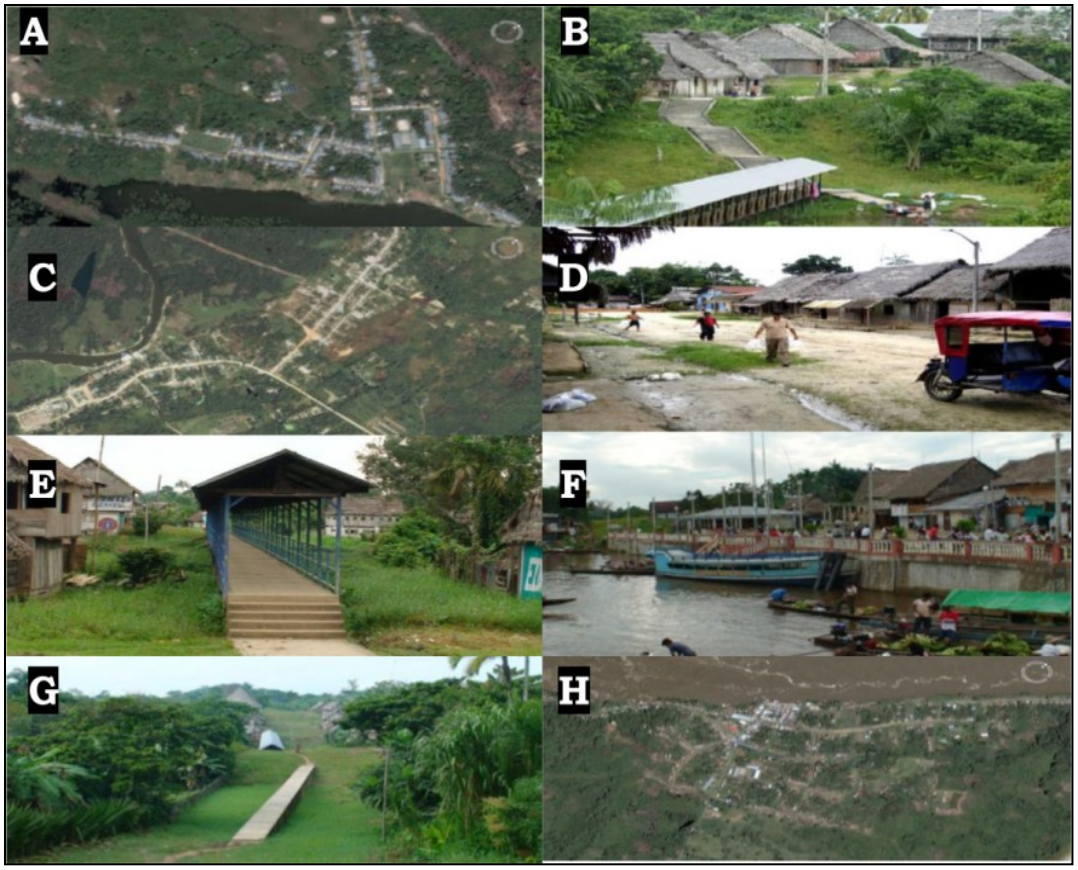

Figure 2.3. Subject villages as visualized by satellite imaging. a: Padrecocha village. b: Santa Rita. c: Santo Tomas & La Union. d: Santo Tomas. e: Mazan. f: Mazan. g: Typical transportation route between villages. h: Mazan.

Table 2.1. Study population characteristics (N=9195) by health posts in the Peruvian Amazon Region of Loreto, 2007.

Health posts [a]	Mazan	Santo Tomas	San Jose de Lupuna	Padrecocha	Total
Variable					
Study Population	3715 (40.4%)	2649 (28.8%)	1247 (13.6%)	1584 (17.2%)	9195
Males	1965 (52.9%)	1395 (52.7%)	663 (53.2%)	814 (51.4%)	4837 (52.6%)
Age 1 (years)					
mean ± SD	23.4 ± 17.9	24.3 ± 18.2	26.5 ± 20.3	24.7 ± 17.0	24.4 ± 18.4
median	18.5	20.1	21.4	21	19.8
Age 2 (years)					
Less than 5	508 (13.7%)	372 (14.0%)	165 (13.2%)	159 (10.0%)	1204 (13.1%)
[5 - 15>	1038 (27.9%)	609 (23.0%)	289 (23.2%)	384 (24.2%)	2320 (25.2%)
[15 - 45>	1635 (44.0%)	1271 (48%)	554 (44.4%)	777 (49.1%)	4237 (46.1%)
45 or older	534 (14.4%)	397 (15.0%)	239 (19.2%)	264 (16.7%)	1434 (15.6%)
Family income					
Nuevos Soles	686.2	340.2	293.8	332.5	483.1
U.S. Dollars	207.9	103.1	89.0	100.8	146.4
Education[b]					
None	108 (5.8%)	61 (4.2%)	48 (6.8%)	24 (2.4%)	241 (4.8%)
Primary	835 (44.8%)	638 (43.5%)	306 (43.4%)	400 (40.2%)	2179 (43.3%)
Secondary	752 (40.3%)	698 (47.6%)	311 (44.1%)	524 (52.6%)	2285 (45.4%)
Tech/Superior	171 (9.2%)	69 (4.7%)	40 (5.7%)	48 (4.8%)	328 (6.5%)
Job[c]					
Loggers	330 (17.0%)	40 (2.7%)	20 (3.0%)	8 (0.8%)	398 (7.8%)
Fishing	37 (1.9%)	89 (6.0%)	16 (2.4%)	100 (10.1%)	242 (4.7%)
Charcoal	0 (0%)	35 (2.3%)	81 (12.0%)	4 (0.4%)	120 (2.4%)
Agriculture	223 (11.5%)	93 (6.2%)	135 (20.0%)	240 (24.1%)	691 (13.5%)
Craftsman	1 (0.1%)	35 (2.3%)	4 (0.6%)	135 (13.6%)	175 (3.4%)
Commerce/Moto	293 (15.1%)	176 (11.8%)	37 (5.5%)	24 (2.4%)	530 (10.4%)
Housewife	590 (30.5%)	467 (31.3%)	238 (35.2%)	296 (29.7%)	1591 (31.2%)
Unemployed	110 (5.7%)	85 (5.7%)	35 (5.2%)	30 (3.0%)	260 (5.1%)
Urbanity Program	9 (0.5%)	16 (1.1%)	2 (0.3)	6 (0.6%)	33 (0.6%)
Other	344 (17.8%)	456 (30.6%)	109 (16.1%)	153 (15.3%)	1062 (20.8%)

[a] Health posts (villages) were: Mazan (Mazan, San Jose, Puerto Alegre), Santo Tomas (Santo Tomas, La Union, Nuevo Quistococha), San Jose de Lupuna (San Jose de Lupuna, Santa Rita, San Pedro, Fray Martin and Padrecocha (Padrecocha, San Andres, Nueva Vida).

[b] Education data excluded all subjects younger than 18 years old.

[c] Job data excluded school students and under school age subjects.

Table 2.2. Malaria prevalence in the study health posts in the Peruvian Amazon Region of Loreto, 2007.

Health post [a]	Mazan	Santo Tomas	San Jose de Lupuna	Padrecocha	Total
Prevalence (95%CI)[b]					
Number of slides	2546	1392	786	832	5556
Any species	2.8% [2.1,3.4]	6.2% [4.9,7.4]	3.9% [2.6,5.3]	2.4% [1.4,3.4]	3.7% [3.2,4.2%]
P. vivax	2.0% [1.5,2.6]	6.2% [4.9,7.4]	2.6% [1.5,3.7]	1.9% [1.0,2.9]	3.1% [2.7,3.6%]
P falciparum	0.7% [0.4,1.0]	0.00	1.3% [0.5,2.1]	0.48% [0.01,0.95]	0.6% [0.4,0.8%]
P vivax: P falciparum	1:0.35	1:0	1:0.5	1:0.25	1:0.19

[a] Health posts (villages) were: Mazan (Mazan, San Jose, Puerto Alegre), Santo Tomas (Santo Tomas, La Union, Nuevo Quistococha), San Jose de Lupuna (San Jose de Lupuna, Santa Rita, San Pedro, Fray Martin and Padrecocha (Padrecocha, San Andres, Nueva Vida).

[b] CI = Confidence interval (exact binomial). Two cases of mixed (*P vivax* plus *P falciparum*) malaria infections were identified and included separately for each malaria parasite species.

Table 2.3. Malaria cases and Annual Parasite Incidence (API) in health establishments study sites from 2003 to 2007.

Health site	Parasite	2003	2004	2005	2006	2007	2004-2007 (≥80%)[d]
SJL[b]	*P vivax*	389	422	660	419	195	EW (2 - 29)
	P falciparum	65	77	413	234	6	2326 / 2880
	API	349.2	383.8	822.8	487.1	152.7	80.8%
MAZ[b]	*P vivax*	1355	1535	1560	1569	1757	EW (2 - 37)
	P falciparum	233	507	764	487	873	8553 / 10640
	API	324.1	416.7	453.6	362.2	529.3	80.4%
PAD[b]	*P vivax*	707	573	1042	385	280	EW (2 - 27)
	P falciparum	62	19	56	22	9	2559 / 3155
	API	480.6	370	445.7	165.1	146.5	81.1%
STO[b]	*P vivax*	944	889	1204	526	169	EW (3 - 29)
	P falciparum	78	35	130	49	18	3276 / 4042
	API[c]	385.7	348.7	503.4	217	70.6	81%

[a] API: Annual Parasite Incidence = confirmed cases during one year / villages population (x 1000)

[b] Health unit located in San Jose de Lupuna (SJL), Mazan (MAZ), Padrecocha (PAD) and Santo Tomas (STO)

[c] Denominator in STO was obtained from the study census in La Union, Nuevo Quistococha and San Tomas

[d] This column indicates the Epidemiological Weeks (EW) interval with at least 80% of the malaria cases considering the last 5 years

Source: Health Regional Directorate of Loreto. STO population was calculated from study census

Table 2.4. Univariate analysis: Epidemiology of malaria in the Peruvian Amazon Region of Loreto (N=9195).

Variables	Some Malaria Episodes		Several Malaria Episodes		P Value
	Odds Ratio	95% CI	Odds Ratio	95% CI	
Age 1 (10yrs)	1.51	1.45, 1.56	1.96	1.88, 2.04	< 0.0001
Age 2 (yrs)					
<5>	1.00[b]		1.00[b]		< 0.0001
[5-15>	3.5	2.92, 4.20	6.96	4.96, 9.76	
[15-45>	7.36	6.17, 8.78	32.49	23.46, 44.99	
[45>	9.15	7.23, 11.57	73.1	51.25, 104.27	
Gender					< 0.0001
Female	1.00[b]		1.00[b]		
Male	1.53	1.38, 1.70	1.52	1.36, 1.70	
Health Establishment					< 0.0001
Mazan	1.00[b]		1.00[b]		
Padrecocha	4.45	3.25, 6.09	10.73	7.78, 14.79	
San Jose de Lupuna	2.79	2.30, 3.37	12.86	10.65, 15.53	
Santo Tomas	2.31	2.05, 2.61	5.38	4.68, 6.18	
Education[c]					< 0.0001
Technical / Superior	1.00[b]		1.00[b]		
Secondary	1.56	1.16, 2.12	2.18	1.58, 3.01	
Primary	1.9	1.40, 2.58	3.59	2.60, 4.97	
None	1.64	1.02, 2.63	3.06	1.91, 4.93	
Job[d]					< 0.0001
Commerce / Mototaxi	1.00[b]		1.00[b]		
Agriculture	4.46	2.98, 6.67	7.79	5.23, 11.60	
Housewife	1.55	1.21, 2.00	1.88	1.46, 2.44	
Craftsman	3.49	1.48, 8.25	6.22	2.71, 14.27	
Charcoal	5.07	1.91, 13.47	18.06	7.15, 45.66	
Loggers	6.99	4.38, 11.15	7.65	4.78, 12.25	
Fishing	3.51	1.91, 6.43	6.8	3.79, 12.21	
None	1.65	1.11, 2.45	1.78	1.19, 2.67	
Urbanity Program	0.78	0.24, 2.61	2.83	1.08, 7.42	
Job outside village					< 0.0001
No	1.00[b]		1.00[b]		
Yes	3.29	2.56, 4.22	4.72	3.69, 6.03	
Travel history 1 (T1)[e]					< 0.0001
No	1.00[b]		1.00[b]		
Yes	2.54	2.05, 3.15	2.79	2.24, 3.47	
Travel history 2(T2)[e]					0.0027
No	1.00[b]		1.00[b]		
Yes	1.3	1.118, 1.50	1.15	0.98, 1.35	
Travel history 3 (T3)[e]					
No	1.00[b]		1.00[b]		< 0.0001
Yes	8.13	4.26, 15.50	5.57	2.91, 10.63	
Living with L/A[f]					< 0.0001
No	1.00[b]		1.00[b]		
Yes	1.238	1.114, 1.376	1.221	1.093, 1.365	

[a] Dependent variable is malaria episodes lifetime as: never (0), some (1-3) and several (≥ 4). Reference is never.
[b] Indicates reference category
[c] Education analysis was performed excluding all subjects younger than 18 years old.
[d] Job analysis was performed excluding school students and under school age subjects.
[e] Iquitos was considered as no travel for T1, T2 & T3. T1 & T2 referred to last month previous to study. T1 for ≥ 10 km or out of village for at least 3 days. T2 for any distance & duration. T3 referred to month previous of at least one of the last four malaria episodes for further than 10km or for at least 3 days. For subjects with never malaria, T3 referred to lifetime.
[f] Living with a person who works on Logging (L) or Agriculture (A).

Table 2.5. Multivariate analysis: Epidemiology of malaria in the Peruvian Amazon Region of Loreto (N=9195).

Variables	Some Malaria Episodes		Several Malaria Episodes		P Value
	Odds Ratio	95% CI	Odds Ratio	95% CI	
Age (10yrs)	1.231	1.149, 1.320	1.576	1.464, 1.697	< 0.0001
Age 2 (yrs)					
<15>	1.00[b]		1.00[b]		<0.0001
[15>	1.537	1.17, 2.02	3.015	2.2, 4.13	
Gender					0.0168
Female	1.00[b]		1.00[b]		
Male	1.167	1.003, 1.358	0.952	0.791, 1.146	
Health Establishment					< 0.0001
Mazan	1.00[b]		1.00[b]		
Padrecocha	10.137	7.219, 14.236	40.563	27.6, 59.614	
San Jose de Lupuna	12.832	10.158, 16.211	127.143	95.756, 168.816	
Santo Tomas	8.636	7.337, 10.166	39.189	31.326, 49.025	
Education					0.054
Technical / Superior	1.00[b]		1.00[b]		
Secondary	1.13	0.786, 1.625	1.322	0.876, 1.994	
Primary	1.123	0.78, 1.616	1.291	0.855, 1.950	
None	0.975	0.582, 1.635	0.725	0.405, 1.298	
Job					< 0.0001
Commerce / Mototaxi	1.00[b]		1.00[b]		
Agriculture	3.212	1.995, 5.174	4.476	2.714, 7.383	
Housewife	1.732	1.263, 2.375	1.659	1.174, 2.343	
Craftsman	1.675	0.679, 4.135	2.14	0.875, 5.238	
Charcoal	1.605	0.587, 4.384	2.208	0.832, 5.859	
Loggers	2.595	1.470, 4.581	4.972	2.746, 9.004	
Fishing	1.164	0.596, 2.273	1.652	0.844, 3.235	
None	1.124	0.69, 1.829	1.133	0.668, 1.923	
Urbanity Program	0.853	0.201, 3.624	2.701	0.689, 10.582	
Student	1.543	1.117, 2.130	1.074	0.749, 1.540	
Under School Age	0.337	0.075, 1.522	0.692	0.146, 3.294	
Other	1.294	0.928, 1.806	1.161	0.808, 1.667	
Travel history (T3)[c]					
No	1.00[b]		1.00[b]		< 0.0001
Yes	31.126	24.717, 39.179	57.818	43.924, 76.107	
Living with L/A[d]					< 0.0001
No	1.00[b]		1.00[b]		
Yes	1.798	1.545, 2.091	2.2	1.85, 2.616	

[a] The dependent variable is number of malaria episodes during lifetime categorized as never (0), some (1-3) and several (≥ 4). The reference category is never.
[b] Indicates reference category.
[c] Iquitos was considered as no travel. T3 referred to month previous of at least one of the last four malaria episodes. For subjects with never malaria, T3 referred to lifetime.
[d] Living with a person who works on Logging (L) or Agriculture (A).
* Adjusted analysis: Polychotomous logistic regression was performed with all variables in the model.

CHAPTER 3: MICROGEOGRAPHICAL DIFFERENCES OF PLASMODIUM VIVAX RELAPSE AND RE-INFECTION IN THE PERUVIAN AMAZON

ABSTRACT

To determine the magnitude of Plasmodium vivax relapsing malaria in rural Amazonia, we carried out a study in four sites in northeastern Peru. Polymerase chain reaction-restriction fragment length polymorphism of PvMSP-3a and tandem repeat (TR) markers were compared for their ability to distinguish relapse versus reinfection. Of 1,507 subjects with *P. vivax* malaria, 354 developed > 1 episode during the study; 97 of 354 (27.5%) were defined as relapse using Pvmsp-3a alone. The addition of TR polymorphism analysis significantly reduced the number of definitively defined relapses to 26 of 354 (7.4%) ($P < 0.05$). Multivariate logistic regression modeling showed that the probability of having > 1 infection was associated with the following: subjects in Mazan (odds ratio [OR] = 2.56; 95% confidence interval [CI] 1.87, 3.51), 15–44 years of age (OR = 1.49; 95% CI 1.03, 2.15), traveling for job purposes (OR = 1.45; 95%CI 1.03, 2.06), and travel within past month (OR = 1.46; 95% CI 1.0, 2.14). The high discriminatory capacity of the molecular tools shown here is useful for understanding the micro-geography of malaria transmission.

INTRODUCTION

An estimated 2.6 billion people live in areas endemic for malaria caused by Plasmodium vivax[1] where this parasite is responsible for a substantial burden of disease accounting for at least 70–80 million clinical cases yearly.[2–5] Plasmodium vivax is the most common form of malaria in the Peruvian Amazon region,[4–6] 32.5% of the population is at risk for malaria and *P. vivax* is responsible for 70–90% of cases in the Peruvian Amazon, with P. falciparum accounting for the remainder.[7] *P. vivax* and *P. falciparum* malaria transmission is characterized in the Peruvian Amazon region as low transmission. Older surveys have found 9.8/1,000 Anopheles spp. vector mosquitoes positive for Plasmodium species, with Anopheles darlingi being the primary vector in the Amazon region.[8,9] Mixed infections with P. falciparum and *P. vivax* are uncommon,[10,11] and asymptomatic malaria parasitemia is present in 3–5% of cross-sectional smears.[12] Published entomological inoculation rates have been reported to range from 0.5 (0.2, 0.8) to 2.5 (1.0, 3.9).[8,13]

Control of vivax malaria at the public health level is complicated by the parasite's unique biological features: early gametocytogenesis, relapsing liver stages, and a wider geographic range caused by tolerance of different climatic conditions.[2] Tools to delineate and quantify *P. vivax* relapses occurring in vivax-endemic regions are key to differentiating relapse from re-infection and to allow for the quantification of the burden of this phenomena, which is known to cause a cumulative lifetime malaria experience of 10–30 episodes.[2] The

biology of *P. vivax* relapse remains poorly understood and is an important obstacle to the public health control of *P. vivax* malaria.[14–16]

Genetic markers have been assessed to discriminate between *P. vivax* infecting strains. Some of the genes identified for this purpose include genes for the circumsporozoite protein (PvCSP) and merozoite surface protein-1 (PvMSP-1),[17] apical membrane protein-1 (PvAMA-1), and merozoite surface protein-3α (PvMSP-3α)[18]; recently, tandem repeat (TR) polymorphism markers[19] and microsatellite methods have been used for this purpose.[20–22] The MSP-3α of *P. vivax* has a molecular weight ranging from 148 to 150 KD, an alanine-rich central domain, and a series of heptad repeats predicted to form a coiled-coil tertiary peptide structure. The TR markers are on a 100 kb chromosomal fragment that includes the *P. vivax* circumsporozoite gene, which is under selective immune pressure and thus is a highly dynamic area of the chromosome. In this study, we used polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of PvMSP-3α (enzymes Hha1 and Alu1) and PCR of nine TR markers. Because of extensive polymorphisms in these markers, it is unlikely that a parasite causing a new infection would possess an identical genotype; the probability of this being the product of individual allele frequencies of each allele of the genes.

This study was designed to test the hypothesis that recurrences caused by relapse are less common than recurrences caused by reinfection. In addition, we explore if a higher level of resolution of *P. vivax* infecting strains during initial and subsequent infections could be carried out by comparing

PvMSP-3α PCR-RFLP versus TR polymorphism analysis in parasites from an initial versus a subsequent *P. vivax* infection, hence allowing relapses to be distinguished from new infections. Such analysis is the key to understanding the transmission dynamics and role of human movement in the maintenance and spread of *P. vivax* in endemic regions.

MATERIALS AND METHODS

Human subject approvals. All patients provided written informed consent to be enrolled in this study, which was approved by the Ethical Committees of Universidad Peruana Cayetano Heredia, Lima, Peru; Asociacion Benefica PRISMA, Lima, Peru; the Directorate of Health of Loreto-Peru; and the Institutional Review Boards of the University of California at San Diego and the Johns Hopkins Bloomberg School of Public Health.

Study sites and duration of follow-up. The field activities of this study were carried out from 2005 to 2008 in northeastern Peru, in the area near the city of Iquitos in the province of Maynas, the capital of the Amazon Department of Loreto. Considering the region's geographical isolation from the rest of Peru, health services within the surrounding areas of Iquitos are relatively good and accessible. Four health posts provide medical services to the study villages (Figure 3.1) described as follows. The Santo Tomas health post is located 16 km from Iquitos with a catchment area of 2,650. The San Jose de Lupuna health post is located 10 km from Iquitos and accessible only by the Nanay River, has a catchment area of 1,250. The Padrecocha health post is located 6 km from Iquitos, is accessible only by the Nanay River, and has a catchment area of 1,800. The Mazan health center is located 50 km northeastern from Iquitos and has a catchment area of 8,000. The duration of follow-up was as follows: 16 months in Padrecocha (from January 2005 to May 2006), 29 months in Santo Tomas and Santo de Lupuna (from February 2005 to July 2007), and 20 months in Mazan (from June 29, 2006 to February 5, 2008).

Blood sampling. Blood samples were collected from patients diagnosed by conventional light microscopy, on site, at the Peruvian Ministry of Health posts according to national norms. Patients identified as infected with only *P. vivax* (microscopy only) were invited to participate in the study. At each enrollment site, venous blood was collected in EDTA Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) and taken to the field project laboratory where samples were aliquoted and frozen at −20°C. Samples were shipped on dry ice to Universidad Peruana Cayetano Heredia, in Lima, where they were stored frozen at −20°C until used for molecular analysis.

Molecular confirmation of *P. vivax* infection. The DNA was extracted from 200 mL of thawed anticoagulated whole blood using the Qiagen Blood Kit (Qiagen, Valencia, CA). Fourteen PCR assays numbered from PCR1 to PCR14 were carried out (Table 3.1). The diagnosis of non-mixed *P. vivax* infections was confirmed in all patients using genus and species-specific nested PCR assays12; samples with mixed infections were omitted from the analysis. The PCR 1, part of a nested PCR (an 18S ribosomal RNA gene fragment specific for genus Plasmodium was used, and a 1,200 basepairs (bp) fragment, allowed for Plasmodium genus-level identification. Identification of *P. vivax*, and *P. falciparum* species was done using PCR2 (a 120 bp fragment specific for *P. vivax*), and PCR3 (a 205 bp fragment specific for *P. falciparum*), respectively.

Molecular genotyping assays. Genotyping of *P. vivax* isolates was performed using two methods: 1) PCR-RFLP analysis of the *P. vivax*

merozoite surface protein-3α (PvMSP3α) gene;[17,18,23] and 2) a PCR assay based on nine previously published tandem repeat (TR) polymorphism markers[19,23] and recently validated in Peru.[23]

***P. vivax* Merozoite Surface Protein-3α.** To assess allelic types of the PvMSP-3α gene, a published PCR-RFLP method was used18 consisting of a nested PCR (PCR4 and PCR5) where a polymorphic fragment of the PvMSP-3α gene was amplified followed by PCR-RFLP (Alu1 and Hha1) as previously described (Table 3.1).[23]

TR polymorphism analysis. We used nine primer pairs previously designed from a 200 kb contiguous DNA segment of 5 *P. vivax* strains from different parts of the world,19 syntenic to *P. falciparum* chromosome 3. We selected these nine primer pairs based on preliminary studies of seven different samples from different areas of the Peruvian Amazon; the other 24 primer pairs tested did not yield at least two alleles.[23] No TR primer pair amplified DNA from either human or P. falciparum.19 The 5 mL of DNA extracted from 200 mL of whole blood and 0.5 mL of primer (Invitrogen, Carlsbad, CA) was added to the PCR mix. The TR markers used in this study were as follows: MN3, MN4, MN12, MN17, MN23, MN24, MN25, MN26, and MN29 (Table 3.1). The PCR electrophoresis and size quantification was done as previously described.[23]

Case definition and data analysis. Recurrence was defined as any subsequent infection regardless of any genotyping pattern. To determine the relapse or reinfection status of all recurrent episodes, the following steps were

followed: First, all PvMSP3α PCR-RFLP patterns of the reference and subsequent infections were identified. Second, the identical patterns from the previous step were analyzed through TR markers using a tolerance of 5% on band sizes as previously described.[23] Relapse was then defined as identical patterns after applying steps 1 and 2 in that order. Reinfection was defined as a difference in reference versus subsequent infection parasites after applying steps 1 and 2. No comparisons were done when mixed or composite infections. The reference was the primary infection for all subjects with 2 episodes. In the case of > 2 episodes, we compared all episodes with the previous ones and if an episode met the criteria then a relapse was considered. On the other hand, because most of the subjects had a past history of vivax malaria, this is true that there would be a relapse of a parasite population that has not been typed because the primary infection has occurred before the study started.

Statistical analysis. Data were analyzed using SAS v.9.1 (SAS, Cary, NC). The significance level for statistical tests was set at $p \leq 0.05$. The risk of recurrence, relapse, and re-infection were estimated using Kaplan-Meier survival analysis. The Cox proportional hazard model was to compare survival functions when assumptions were met.

RESULTS

Enrollment, baseline characteristics, and *P. vivax* recurrence rates. After a baseline health assessment, 1,507 subjects with *P. vivax* malaria were enrolled in the study. Of these, 575 (38.2%) were enrolled in Santo Tomas, 198 (13.1%) in San Jose de Lupuna, 386 (25.6%) in Padrecocha, and 348 (23.1%) in Mazan. Eight hundred and thirty-one (831) (55.1%) were male, 676 (44.8%) female (Table 3.2).

Recurrences of *P. vivax* infections after Chloroquine (CQ)-Primaquine (PQ) treatment were relatively common in the Peruvian Amazon. Despite the provision of standard therapy for *P. vivax* including CQ and PQ (0.5 mg/kg/day for 7 days) for all patients according to Peruvian Ministry of Health policy, during the study period, 354 out of 1,507 subjects (23.5%) developed at least one subsequent vivax malaria episode. Of these, 274 developed 1 subsequent episode, 60 developed 2 subsequent episodes, 15 developed 3 subsequent episodes, 4 developed 4 subsequent episodes, and 1 subject developed 5 subsequent episodes. The proportion of subjects who remained free of recurrence at the end of the follow up were 75.8% in San Jose Lupuna, 77.6% in Santa Tomas, 61.8% in Mazan, and 88.6% in Padrecocha (Table 3.2). Overall, there was a significantly higher risk of recurrences in Mazan compared with other villages (Santo Tomas, San Jose de Lupuna, and Padrecocha) ($c2 = 54.6$, 1 degrees of freedom [df], $p < 0.0001$). Such differences were caused by differences in reinfection rates ($c2 = 18.95$, 1 df, $p < 0.0001$) rather than relapse rates ($c2 = 0.17$, 1 df, $p = 0.68$), which is

expected provided that treatment is identical and that directly observed therapy was deployed in study sites.

PCR-RFLP and TR analysis of the Pvmsp-3a gene. The Hha I and Alu I PCR-RFLP assays showed easily distinguishable restriction patterns in all samples. The PCR-RFLP patterns showed 500–600 bp for Alu I digests and the largest fragments between 950–1,100 bp for Hha I digests. These fragments and the smaller ones from 150–750 bp were analyzed by RFLP.

The RFLP analysis of MSP3-a PCR fragment produced 15 patterns after Hha I digestion (Table 3.3). From these 15 patterns, patterns 5, 11, 12, 14, and 15 were complex patterns; therefore, considered as mixed or composite *P. vivax* parasite samples, not unique patterns, because the sum of the restriction fragments exceeded the size of the primary product. Therefore, based on restriction patterns from digestion of PCR products with Hha I, 10 allele variants have been detected among the 354 patient-isolates (Figure 3.2, Table 3.3).

The RFLP analysis of PvMSP3-a PCR fragment produced 14 patterns after Alu I digestion. From these 14 patterns, patterns 6 and 10 were complex patterns; therefore, considered as mixed or composite *P. vivax* parasite samples, because the sum of the restriction fragments exceeded the size of the primary product. Therefore, based on restriction patterns from digestion of PCR products with Alu I, 12 allele variants have been detected among the 354 patient isolates (Table 3.4).

The overall diversity of *P. vivax* was determined on the basis of TR genotyping for the primary samples obtained from all study villages. The genetic diversity of each TR locus was 0.54 (MN3), 0.58 (MN24), 0.60 (MN17), 0.70 (MN4), 0.73 (MN12), 0.73 (MN19), 0.87 (MN23), 0.88 (MN25), and 0.88 (MN26) (Table 3.5, Figure 3.3). The allelic frequencies at each TR locus are shown in Figure 3.4.

Recurrences of *P. vivax*. Of the 1,507 subjects enrolled with *P. vivax* malaria infection, 354 (23.5%) developed more than one vivax malaria episode; 1,153 subjects did not have another vivax malaria episode during the period of the study. Regarding the ability to differentiate between *P. vivax* genotypes, TR polymorphism analysis differentiated a higher proportion of subsequent infections than PCR-RFLP analysis alone ($p < 0.05$).

The odds of those enrolled with > 1 episode of *P. vivax* malaria, having increased lifetime malaria cases, compared with those who were enrolled with only one episode of *P. vivax* malaria, were 1.1 times higher for subjects within 5–14 years of age ($p < 0.05$) (95% CI 1.03, 1.19), and 1.05 times higher for subjects within 15–45 years of age ($p < 0.05$) (95% CI 1.01, 1.08) (Tables 3.6 and 3.7). The odds of developing > 1 episode of *P. vivax* malaria whether caused by relapse or reinfection were 2.6 times higher in the more remote village of Mazan than in villages closer to Iquitos ($p < 0.001$) (odds ratio [OR] = 2.6, 95% confidence interval [CI] 2.0, 3.4). There was no difference in age, gender, level of education, and time living in the village with respect to the odds of developing a subsequent vivax episode (Table 3.2). Significantly,

occupation related travel from a village (self-reported by the subject) was associated with a 2-fold elevated odds of developing more than one vivax malaria episode (whether caused by relapse or reinfection) during the study period (OR = 2.0, 95% CI 1.5, 2.6; $p < 0.05$) (Tables 3.2 and 3.8). People who developed a recurrent vivax malaria episode during the period of the study had a significantly higher median number of cumulative lifetime total of self-reported malaria cases than people who only developed one vivax episode in the study period ($p < 0.001$) (Table 3.2).

The odds of developing > 1 episode of *P. vivax* malaria, whether caused by relapse or reinfection, were 2.6 times higher for subjects who had traveled in the prior month to any of their last four malaria episodes than in subjects without such travel ($p < 0.05$) (95% CI 1.9, 3.6) (Tables 3.2 and 3.8). The odds of developing > 1 episode of *P. vivax* malaria, whether caused by relapse or reinfection, were 2.5 times higher for subjects who had traveled > 10 Km and > 3 days in the prior month to episode than in subjects without such travel ($p < 0.05$) (95% CI 1.8, 3.5) (Tables 3.2 and 3.8). Household variables (exterior/interior wall materials, locations of kitchen, roof material, number of bed nets per bed, possessions) were found not to be different in any comparison between subjects with only 1 episode, subjects with > 1 episode, subjects with relapse, and subjects with reinfection) (Table 3.8). Multivariate logistic regression modeling probability of > 1 infection during study period showed that odds for subjects in Mazan (OR = 2.56), 15–44 years of age (OR = 1.5), traveling for job purposes (OR = 1.5), with increased

lifetime malaria cases (OR = 1.1), and who traveled in the month before the malaria episode (OR = 1.5), were significantly higher than subjects without such profile (95% CIs 1.9, 3.5; 1.03, 2.2; 1.03, 2.1; 1.04, 1.1; 1.0, 2.1, respectively) (Table 3.9).

Timing of *P. vivax* recurrences. The median time interval between laboratory-confirmed *P. vivax* infections was 258.6 days in Santa Tomas, 244.5 days in San Jose Lupuna, 230.7, and 131.7 days in Mazan. More than a half of the recurrences were diagnosed up to 240 days: 54% in Santa Tomas, 60% in San Jose Lupuna, 56% in Padrecocha, and 85% in Mazan. Most of the recurrences were diagnosed up to 365 days after drug treatment: 72% in Santa Tomas, 75% in San Jose Lupuna, 74% in Padrecocha, and 97% in Mazan. Only five recurrences (two of them in Mazan, and one in each of the other sites) were diagnosed up to 28 days after treatment, and only one out of these five recurrences was found to be a relapse.

Comparison of relapse versus re-infection using PCR-RFLP versus TR markers. The median number of times having traveled in the month previous to vivax malaria diagnosis was significantly higher in subjects who developed re-infections compared with the ones who relapsed with an identical genotype ($p < 0.05$) (Table 3.6).Regarding developing a subsequent relapse or re-infection, there was no difference between people who reported traveling further than 10 Km and for 3 days in the month before diagnosis and people who did not travel (OR = 2.2, 95% CI 0.9, 5.4; $p = 0.38$) (Table 3.6). Regarding developing a subsequent relapse or re-infection, there was no difference

between people who travel out of village for work and people who do not (OR = 1.0, 95% CI 0.4, 2.5; p = 0.39) (Table 3.6). Of 814 infections, the different rates of multiple *P. vivax* genotypes were: 131 (16.1%) by TR-PCR, 37 (4.5%) by PvMSP-3α PCR-RFLP, and 153 (18.8%) if both typing schemes were used together.

Comparison of time intervals to relapse and to re-infection in all villages together. Using only PvMSP-3α markers, the risk of relapse was 1.5 higher than the risk of re-infection (rate ratio [RR] = 1.5, 95% CI 1.2, 2.0; p < 0.05). Adding TR markers, the risk of relapsing was 1.7 higher than the risk of a re-infection (RR = 1.7, 95% CI 1.2, 2.5; p < 0.05) (Figure 3.5).

Comparison of time intervals to relapse and to re-infection in Mazan. In Mazan, using PvMSP-3a markers, the time to relapse versus re-infection showed a strong but not significant trend and was borderline not different (RR = 1.4, 95% CI 1.0, 2.0; p = 0.06). Adding TR markers, similar results were found (RR = 1.6, 95% CI 1.0, 2.7; p = 0.07).

Comparison of time intervals to relapse and to re-infection in the other villages. In the non-Mazan villages, which are demographically different than Mazan, PvMSP-3α markers indicated that time intervals between relapse and re-infection were borderline significantly different. The relative risk of relapse was 1.47 times higher than re-infection (RR = 1.47, 95% CI 1.11, 1.94; p < 0.05). Adding TR markers, the risk of relapse was not different than the risk of a re-infection (p = 0.18).

Comparison of time intervals to relapse in Mazan versus the other villages. People in Mazan were 2.4 times more likely to develop a recurrent *P. vivax* malaria caused by relapse (not caused by re-infection) than people in the other villages of the study (RR = 2.4, 95% CI 1.1, 5.5; p = 0.03).

DISCUSSION

In a large, prospective study of *P. vivax* infection in diverse transmission settings in the Peruvian Amazon, the use of new molecular tools identified a high diversity of *P. vivax* parasites. Furthermore, combining the use of these molecular tools with socio-demographic analysis delineated a differential risk of relapse versus re-infection in distinct microgeographical settings within the Loreto region. These data are important to guide future surveillance and malaria intervention and control efforts not only in Peru but globally.

Molecular comparison of *P. vivax* parasites associated with relapses and those associated with the respective primary infections has been reported previously in a small number of patients: 6 patients in Canada who had acquired the infection in different areas of endemicity,[16] 10 patients in Brazil,[24] and five patients in Thailand.[25] Parasite diversity was assessed using molecular markers in the first two studies (pvcs and pvmsp1, and pvmsp-1 alone, respectively), whereas a panel of monoclonal antibodies was used in the third study. Although it was concluded that parasites associated with primary infection and those associated with relapse are usually of a similar genetic composition, evidence for novel genotypes in *P. vivax* associated with relapses was obtained for 24% of the patients (one of six patients in Canada,[18] 2 of 10 patients in Brazil, and 2 of 5 patients in Thailand.[25] Another study showed relapses in 107 subjects (28 [78%] in Thailand, 55 [73%] in Myanmar, and 24 [63%] in India) with recurrent infections harboring none of the parasites

that caused the primary infection; and concluded that activation of heterologous hypnozoite populations is the most common cause of first relapse in *P. vivax* malaria.[14]

This study was carried out to assess relapse versus reinfection in the Amazonian hypoendemic malaria setting by combining two molecular tools to characterize multiple episodes of *P. vivax* malaria. Given the high degree of internal mobility of people in the region, well-validated tools to determine the microgeographic characteristics of *P. vivax* geospatial transmission dynamics within the region will be essential for future malaria control efforts.

Previous studies have used molecular markers to distinguish relapse from reinfection but failed to take into account the field context of travel, comparison of villages, etc. Low transmission intensity but with high diversity is also an important finding of our study. Although some recrudescences could result from inadequate drug absorption or unusual disposition kinetics, recurrent infections in this study were newly acquired infections or relapses.[26,27]

The Pvmsp-3α polymorphism analysis has been an important molecular epidemiological tool for distinguishing *P. vivax* infections in malaria-endemic areas.[18,28,29] This study used Pvmsp-3α PCR-RFLP and TR-PCR analysis as tools for differentiating primary from subsequent *P. vivax* infections. We found that the TR markers alone and in combination with the independent Pvmsp-3α locus were more highly discriminatory.

The TR markers used in this study are on a highly dynamic 100 kb chromosomal region where selective pressure is thought to be exerted because of the presence of the Pvcsp gene. Despite vivax malaria being hypoendemic at the population level in the Peruvian Amazon, considering that our study was done in a limited geographic area and time frame, in contrast to previous reports,[21,30,31] our findings are consistent with a relatively high degree of *P. vivax* diversity in the Peruvian Amazon villages near to Iquitos city.

By Pvmsp-3α PCR-RFLP analysis, 12 different allelic variants were detected using the AluI PCR-RFLP and 11 allelic variants using HhaI. Using TR polymorphisms to analyze the same samples, a much greater degree of diversity was found allowing for greater discrimination within the patient groups. These data indicate that even within a region of hypoendemicity where little parasite outbreeding might be present, TRs are more useful and sensitive than the Pvmsp-3α markers. Previous studies have concluded that in some regions *P. vivax* populations emerging from hypnozoites commonly differ from the populations that caused the acute episode and that activation of heterologous hypnozoite populations is the most common cause of first relapse in patients with vivax malaria.[16] Our data are consistent with this finding, because some of the apparent re-infections we found could be caused by relapse of a different clone acquired at the time of a primary infection or even afterward. Therefore, there is an inherent limitation of molecular epidemiological methods to differentiate infecting strains of *P. vivax*,

highlighting the need of further understanding of the nature of relapses[32] and the development of alternatives to PQ for radical treatment of hypnozoites.[33] We have shown that by adding more polymorphic markers into the analysis, the proportion if identical genotypes may be even lower than what we found with msp3α. For example, a similar study in Brazil[34] has found very few 14-marker haplotypes in recurrent *P. vivax* infections using microsatellite typing. In this study, we considered a 5% tolerance in band sizes for TR markers to consider the possibility that relapsing parasites may be a minor, genetically different parasite clone that could have missed when typing parasites at the reference infection. Moreover, as shown in previous studies, we found 101 distinct molecular profiles among 110 patients meaning that here is a high degree of *P. vivax* diversity as demonstrated by our pool of TR markers,[23] therefore, this is very unlikely that non-related parasites may have been considered identical after applying our 2-step tool of genotyping.

Relapse versus re-infection depending on socio-demographic context has important policy implications. An important finding arising from this study is that people in a rural area of the Peruvian Amazon remote from the main city of Iquitos (Mazan) were significantly more likely to develop a subsequent episode caused by relapse (not to re-infection) than people in other villages where the socio-demographic context is different. Peruvian Ministry of Health policy indicates that anti-malarial drug therapy is to be directly observed. We suspect that the higher mobility of the more rural population (i.e., travel away from village for occupational reasons such as

logging and forest extraction activities) make it likely both that patients might not receive the standard anti-*P. vivax* treatments compliantly, and also that this group of people might more likely to be re-infected. Other possible explanations of such phenomena could also be that relapse is stimulated by some activities or mechanisms related to the places where people travel or by mosquito bites.

In 2001, adherence problems to PQ led the Peruvian National Malaria Control Program to shorten the length of the treatment from a 14- to 7-day PQ course, but increasing the daily dose from 0.25 to 0.5 mg/kg/day. It was reported at the ASTMH 58th Annual Meeting that 7 days of PQ plus CQ proved as effective as the usual 14-day regimen. Another study by Solari and others[35] during 1998–1999 found there was no difference between 7 days (10%) and 14 days (6.6%) of PQ. In this study, we found only 5 out of 354 subjects (1.4%) who experienced a recurrence during 28 days after treatment and no clinical resistance to malaria; therefore, we think resistance is not a problem with current treatment but drug resistance surveillance and research for new drugs are needed.

The data presented here showed an unexpectedly high proportion of infections caused by more than one parasite genotype. This is particularly remarkable given our current knowledge of transmission intensity and entomological inoculation rates in the Loreto region, and suggests that we lack critical knowledge of the micro-geography of malaria transmission at the actual places where people acquire their *P. vivax* infections. When two or more

different genotypes or clones co-infect an individual or mosquito, cross-strain fertilization and genetic recombination are more likely to occur; therefore, it is quite possible that there is a higher risk subpopulation that makes a disproportionate contribution to the generation of high *P. vivax* genetic diversity. The present molecular epidemiology study and its development of high resolution tools provide new insights into the relationship of socio-demographic characteristics of malaria transmission dynamics in a low transmission/malaria hypoendemic region.

ACKNOWLEDGMENTS

We thank Paula Maguina for her research contributions that were essential to assure regulatory compliance, ethics, and logistical aspects of this project, and Amanda Rondinelli and Shira Abeles for their insightful discussions.

I would like to acknowledge co-authors Pablo Penataro, Kimberly Brouwer, Manuel Fasabi, Maritza Calderon, Sonia Torres, Robert Gilman, and Margaret Kosek, for their contribution to this paper and permission to use it as part of my dissertation

REFERENCES

1. Guerra CA, Snow RW, Hay SI, 2006. Mapping the global extent of malaria in 2005. *Trends Parasitol* 22: 353–358.

2. Mendis K, Sina BJ, Marchesini P, Carter R, 2001. The neglected burden of Plasmodium vivax malaria. *Am J Trop Med Hyg* 64: 97–106.

3. Baird JK, 2007. Neglect of Plasmodium vivax malaria. *Trends Parasitol* 23: 533–539.

4. Price RN, Tjitra E, Guerra CA, Yeung S, White NJ, Anstey NM, 2007. Vivax malaria: neglected and not benign. *Am J Trop Med Hyg* 77: 79–87.

5. Hay SI, Guerra CA, Tatem AJ, Noor AM, Snow RW, 2004. The global distribution and population at risk of malaria: past, present, and future. *Lancet Infect Dis* 4: 327–336.

6. Duarte EC, Gyorkos TW, Pang L, Abrahamowicz M, 2004. Epidemiology of malaria in a hypoendemic Brazilian Amazon migrant population: a cohort study. *Am J Trop Med Hyg* 70: 229–237.

7. Pan American Health Organization, Health Information and Analysis, 2008. Health Situation in the Americas: Basic Indicators 2008. Washington, DC.

8. Flores-Mendoza C, Fernandez R, Escobedo-Vargas KS, Vela-Perez Q, Schoeler GB, 2004. Natural Plasmodium infections in Anopheles darlingi and Anopheles benarrochi (Diptera: Culicidae) from eastern Peru. *J Med Entomol* 41: 489–494.

9. Schoeler GB, Flores-Mendoza C, Fernandez R, Davila JR, Zyzak M, 2003. Geographical distribution of Anopheles darlingi in the Amazon Basin region of Peru. *J Am Mosq Control Assoc* 19: 286–296.

10. Branch O, Casapia WM, Gamboa DV, Hernandez JN, Alava FF, Ronca N, Alvarez E, Perez EJ, Gotuzzo E, 2005. Clustered local transmission and asymptomatic Plasmodium falciparum and Plasmodium vivax malaria infections in a recently emerged, hypoendemic Peruvian Amazon community. *Malar J* 4: 27.

11. Branch OH, Takala S, Kariuki S, Nahlen BL, Kolczak M, Hawley W, Lal AA, 2001. Plasmodium falciparum genotypes, low complexity of

infection, and resistance to subsequent malaria in participants in the Asembo Bay Cohort Project. *Infect Immun* 69: 7783–7792.

12. Roshanravan B, Kari E, Gilman RH, Cabrera L, Lee E, Metcalfe J, Calderon M, Lescano AG, Montenegro SH, Calampa C, Vinetz JM, 2003. Endemic malaria in the Peruvian Amazon region of Iquitos. *Am J Trop Med Hyg* 69: 45–52.

13. Vittor AY, Gilman RH, Tielsch J, Glass G, Shields T, Lozano WS, Pinedo-Cancino V, Patz JA, 2006. The effect of deforestation on the human-biting rate of Anopheles darlingi, the primary vector of falciparum malaria in the Peruvian Amazon. *Am J Trop Med Hyg* 74: 3–11.

14. Imwong M, SnounouG, Pukrittayakamee S, TanomsingN,KimJR, Nandy A, Guthmann JP, Nosten F, Carlton J, Looareesuwan S, Nair S, Sudimack D, Day NP, Anderson TJ, White NJ, 2007. Relapses of Plasmodium vivax infection usually result from activation of heterologous hypnozoites. *J Infect Dis* 195: 927–933.

15. Srivastava HC, Sharma SK, Bhatt RM, Sharma VP, 1996. Studies on Plasmodium vivax relapse pattern in Kheda district, Gujarat. *Indian J Malariol* 33: 173–179.

16. Craig AA, Kain KC, 1996. Molecular analysis of strains of Plasmodium vivax from paired primary and relapse infections. *J Infect Dis* 174: 373–379.

17. Cui L, Escalante AA, Imwong M, Snounou G, 2003. The genetic diversity of Plasmodium vivax populations. *Trends Parasitol* 19: 220–226.

18. Bruce MC, Galinski MR, Barnwell JW, Snounou G, Day KP, 1999. Polymorphism at the merozoite surface protein-3alpha locus of Plasmodium vivax: global and local diversity. *Am J Trop Med Hyg* 61: 518–525.

19. Feng X, Carlton JM, Joy DA, Mu J, Furuya T, Suh BB, Wang Y, Barnwell JW, Su XZ, 2003. Single-nucleotide polymorphisms and genome diversity in Plasmodium vivax. *Proc Natl Acad Sci* 100: 8502–8507.

20. Gomez JC, McNamara DT, Bockarie MJ, Baird JK, Carlton JM, Zimmerman PA, 2003. Identification of a polymorphic Plasmodium vivax microsatellite marker. *Am J Trop Med Hyg* 69: 377–379.

21. Karunaweera ND, Ferreira MU, Munasinghe A, Barnwell JW, Collins WE, King CL, Kawamoto F, Hartl DL, Wirth DF, 2008. Extensive microsatellite diversity in the human malaria parasite Plasmodium vivax. *Gene* 410: 105–112.

22. Nyachieo A, VAN Overmeir C, Laurent T, Dujardin JC, D'Alessandro U, 2005. Plasmodium falciparum genotyping by microsatellites as a method to distinguish between recrudescent and new infections. *Am J Trop Med Hyg* 73: 210–213.

23. Kosek M, Yori P, Gilman RH, Calderon M, Zimic M, Chuquiyauri R, Jeri C, Pinedo V, Matthias MA, Llanos-Cuentas A, Vinetz JM, 2012. High degree of Plasmodium vivax diversity in the Peruvian Amazon demonstrated by tandem repeat polymorphism analysis. *Am J Trop Med Hyg* 86: 580 –586.

24. Kirchgatter K, del Portillo HA, 1998. Molecular analysis of Plasmodium vivax relapses using the MSP1 molecule as a genetic marker. *J Infect Dis* 177: 511–515.

25. Khusmith S, Tharavanij S, Bunnag D, 1998. Antigenic disparity of Plasmodium vivax causing initial symptoms and causing relapse. *Southeast Asian J Trop Med Public Health* 29: 519–524.

26. Pukrittayakamee S, Chantra A, Simpson JA, Vanijanonta S, Clemens R, Looareesuwan S, White NJ, 2000. Therapeutic responses to different antimalarial drugs in vivax malaria. *Antimicrob Agents Chemother* 44: 1680 –1685.

27. Pukrittayakamee S, Imwong M, Looareesuwan S, White NJ, 2004. Therapeutic responses to antimalarial and antibacterial drugs in vivax malaria. *Acta Trop* 89: 351–356.

28. Zakeri S, Barjesteh H, Djadid ND, 2006. Merozoite surface protein-3alpha is a reliable marker for population genetic analysis of Plasmodium vivax. *Malar J* 5: 53.

29. Bruce MC, Galinski MR, Barnwell JW, Donnelly CA, Walmsley M, Alpers MP, Walliker D, Day KP, 2000. Genetic diversity and dynamics of Plasmodium falciparum and *P. vivax* populations in multiply infected children with asymptomatic malaria infections in Papua New Guinea. *Parasitology* 121: 257–272.

30. Leclerc MC, Durand P, Gauthier C, Patot S, Billotte N, Menegon M, Severini C, Ayala FJ, Renaud F, 2004. Meager genetic variability of the human malaria agent Plasmodium vivax. *Proc Natl Acad Sci* 101: 14455–14460.

31. Chen N, Auliff A, Rieckmann K, Gatton M, Cheng Q, 2007. Relapses of Plasmodium vivax infection result from clonal hypnozoites activated at predetermined intervals. *J Infect Dis* 195: 934–941.

32. Collins WE, 2007. Further understanding the nature of relapse of Plasmodium vivax infection. *J Infect Dis* 195: 919–920.

33. Galappaththy GN, Omari AA, Tharyan P, 2007. Primaquine for preventing relapses in people with Plasmodium vivax malaria. *Cochrane Database Syst Rev* CD004389.

34. Orjuela-Sanchez P, da Silva NS, da Silva-Nunes M, Ferreira MU, 2009. Recurrent parasitemias and population dynamics of Plasmodium vivax polymorphisms in rural Amazonia. *Am J Trop Med Hyg* 81: 961–968.

35. Lely Solari-Soto, Alonso Soto-Tarazona, Daniel Mendoza-Requena, Alejandro Llanos-Cuentas, 2002. Ensayo clinico del tratamiento de la malaria vivax con esquema acortado de primaquina comparado con el esquema tradicional. *Rev Per Soc Med Intern* 15:4.

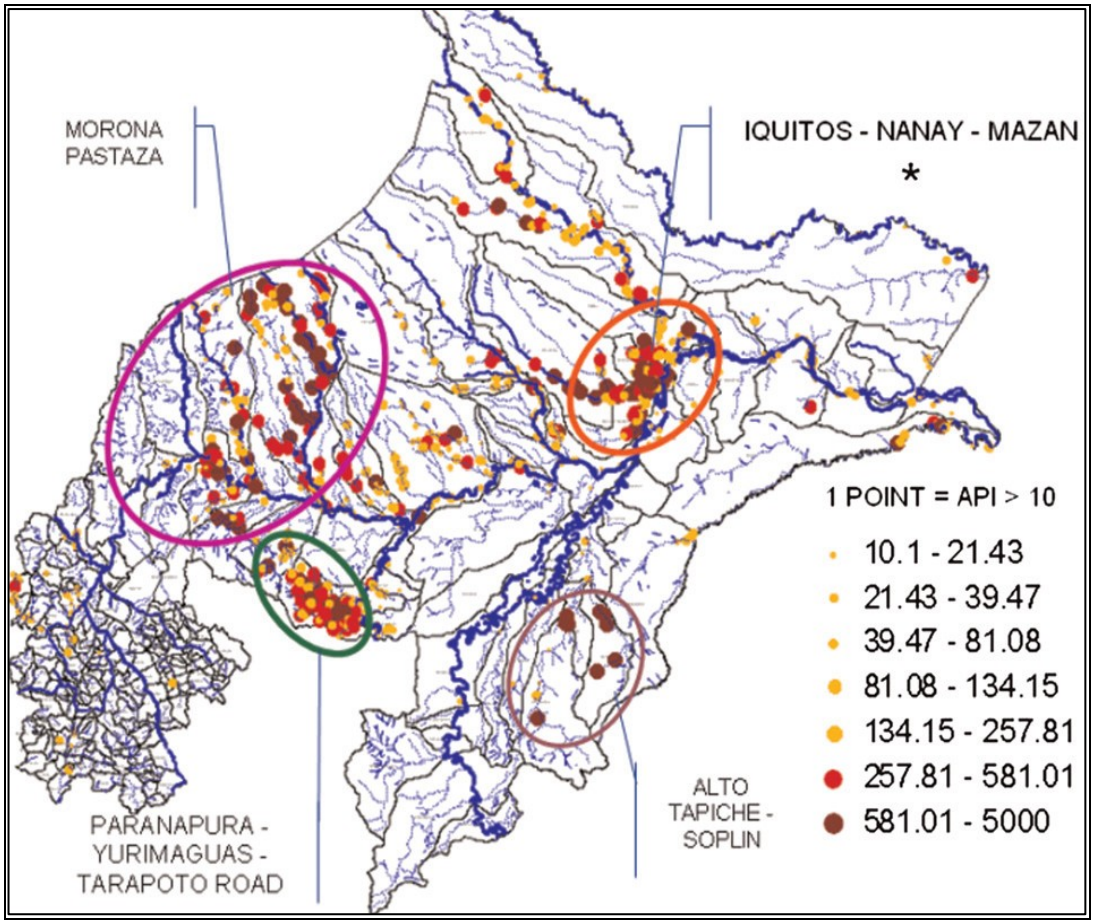

Figure 3.1. Location of study sites in surrounding areas of Iquitos, Peruvian Amazon (*). Axes of Malaria transmission in Loreto, Peru 2007. Sources: Loreto Health Regional Directorate, Malaria Control in Border Areas of the Andean Region (PAMAFRO).

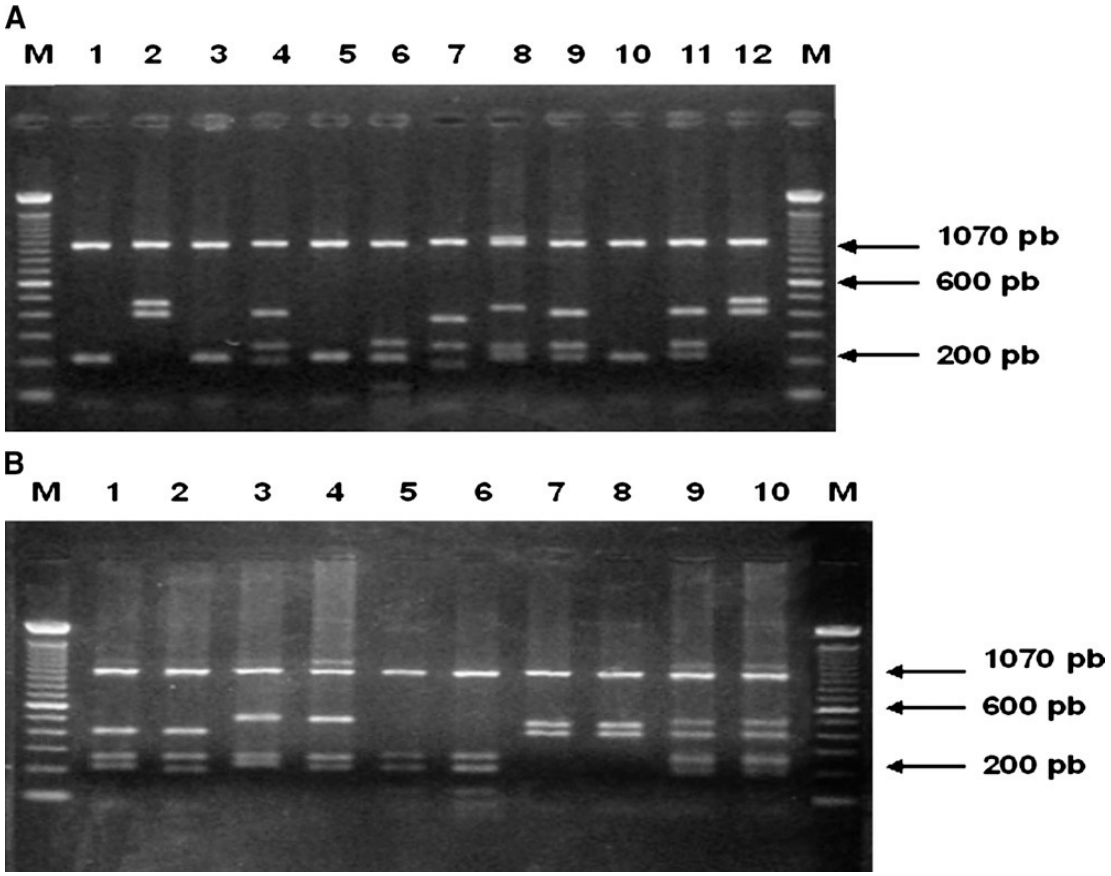

Figure 3.2. Patterns after digestion with restriction endonuclease enzyme HhaI the gen coding MSP3a. from *P. vivax* (M: 100bp DNA ladder) (A) Lines 1, 3, 5, and 10, pattern 1; line 6 pattern 2; line 7 pattern 3; line 8 pattern 4; line 4, 9, and 11 pattern 5; line 2 and 12 pattern 7. (B) Lines 1 and 2 pattern 6; lines 7 and 8 pattern 7; lines 3 and 4 pattern 8; lines 9 and 10 pattern 9; lines 5 and 6 pattern 2.

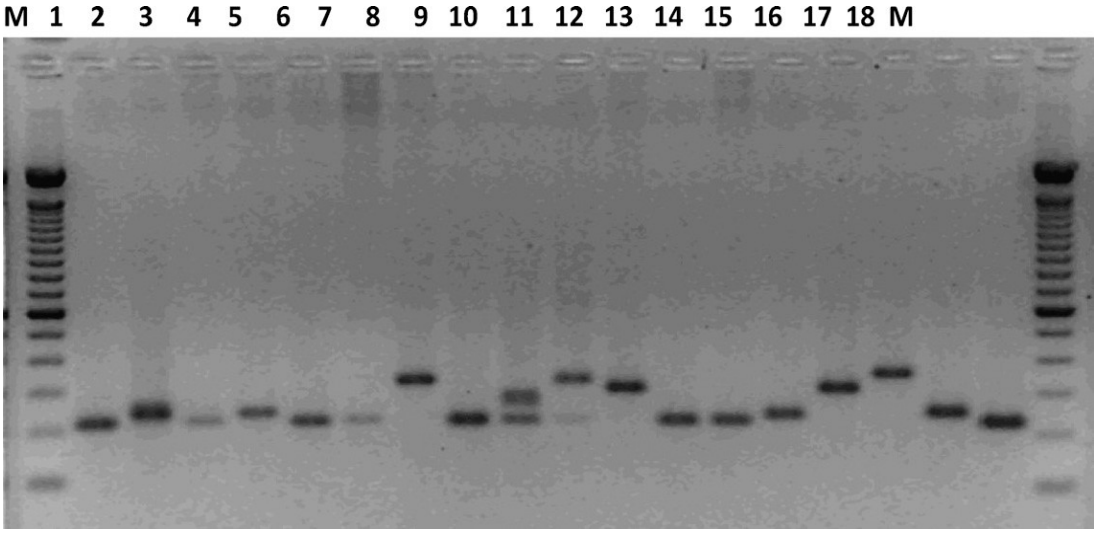

Figure 3.3. Tandem repeat (TR) marker MN23 has 13 allelic types, e.g., allelic types: 1(227), 2(244), 345(227), 6(244), 7(227), 8(227), 9(315), 10(227), 11(265/227), 12(330/227), 13(310), 14(227), 15(227), 16(244), 17(315), and 18(350). Band sizes for TR markers were estimated by automated computational analysis software using a 100 bp ladder (M) on each side of the gel as standard.

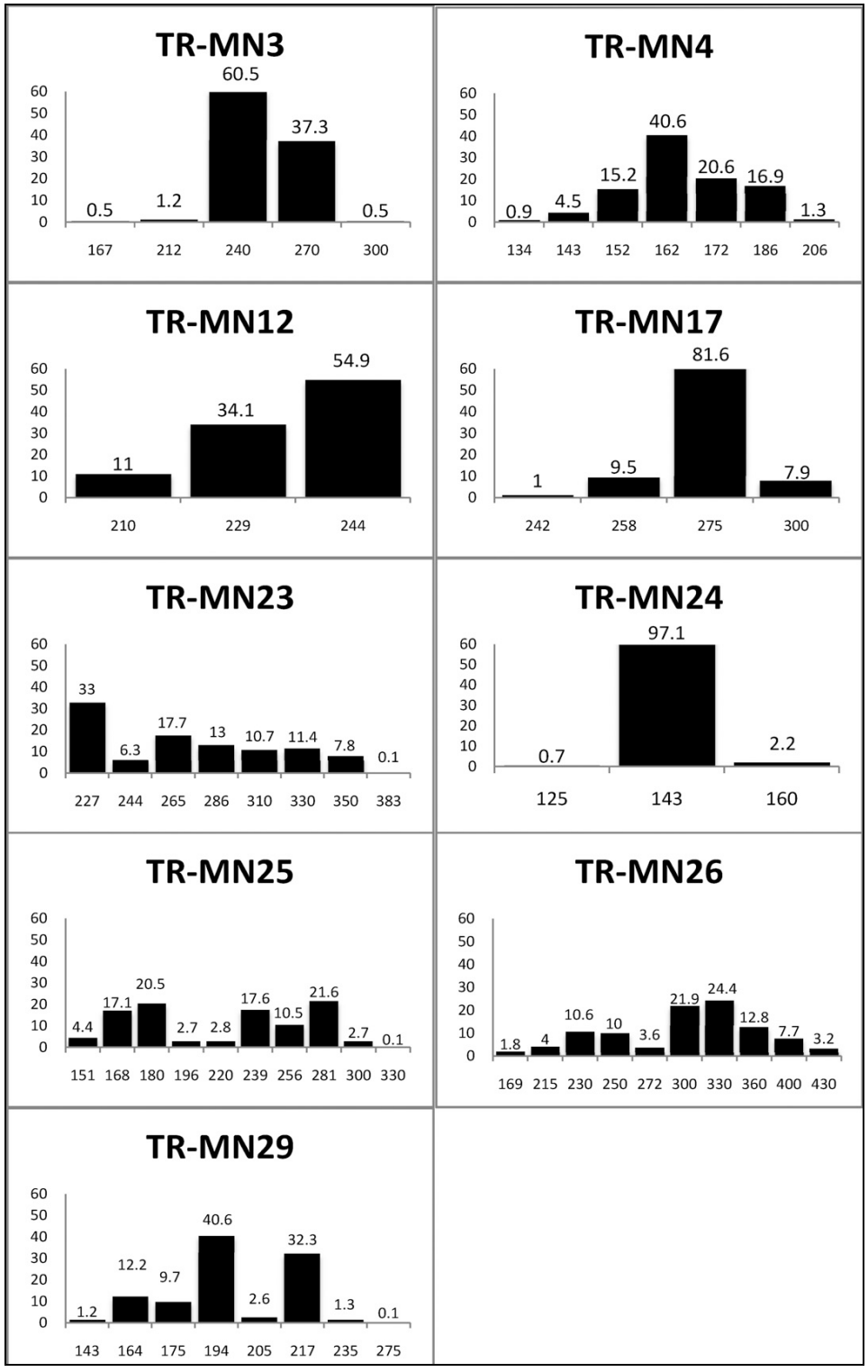

Figure 3.4. Proportion of alleles of line tandem repeat (TR) markers in 814 case samples of Plasmodium vivax malaria.

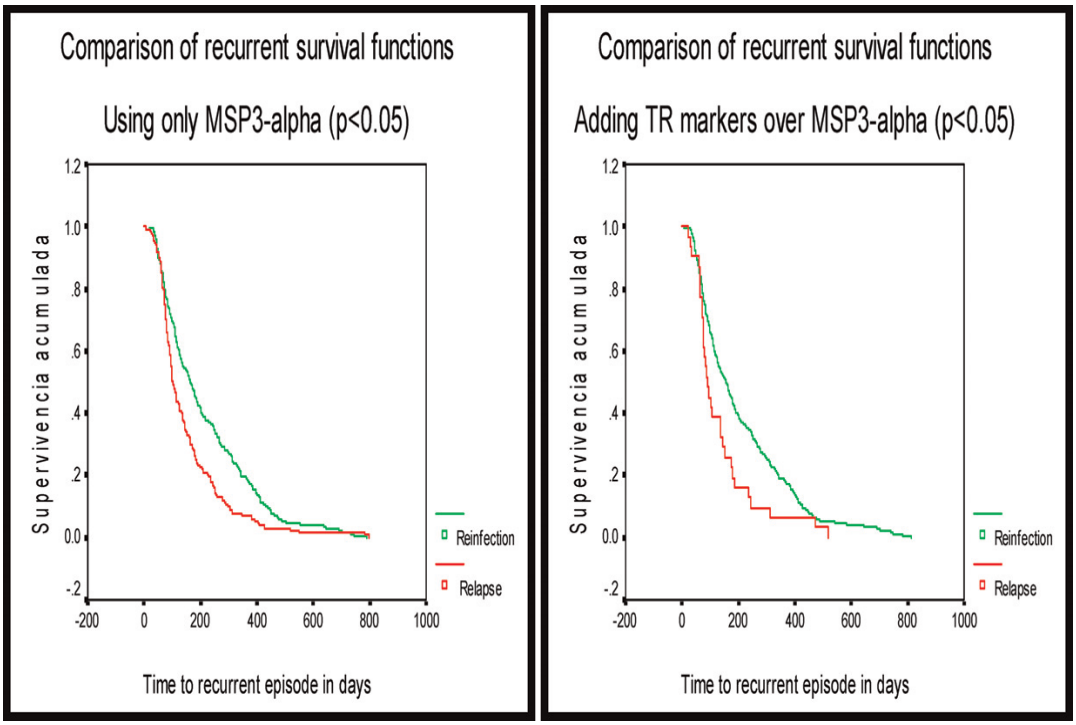

Figure 3.5. Comparison of recurrent survival functions for relapses (green solid line) and re-infections (red solid line) genotyping outcomes. Survival analysis techniques were used to compare the time interval for recurrent infections in relapsing and re-infection episodes, (A) Based on Merozoite Surface Protein-3α (MSP-3α) Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) only and (B) adding the tandem repeat (TR) markers. In both cases the difference was statistically significant ($P < 0.05$) meaning there is a significant difference in the two survival recurrent functions when compared using either MSP-3α only or both MSP-3α/TR markers. Analysis was done using Cox proportional hazard regression model.

Table 3.1. Primers, polymerase chain reaction (PCR) mix and cycling conditions used for *Plasmodium vivax* confirmation and genotyping using Merozoite Surface Protein 3α (MSP3α) Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP), and tandem repeat (TR) markers.

Plasmodium vivax confirmatory nested PCR (PCR1: Genus PCR; PCR2 and 3: Species PCR)		
Primer Sequence* (forward/reverse)	PCR-Mix†	PCR-Cycling‡
PCR1: P1/P2: CTTGTTGTTGCCTTAAACTTC/ TTAAAATTGTTGCAGTTAAAACG PCR2: V1/V2: CGCTTCTAGCTTAATCCACATAACTGATAC/ ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA PCR3: F1/F2: TTAAACTGGTTTGGGAAAACCAAATATATT/ ACACAATGAACTCAATCATGACTACCCGTC	2 mM $MgCl_2$, 200 µM dNTPs, 0.25 µM P1/P2, 0.3 U Taq-p§, 3 µL DNA. 1 µL product of PCR1, 0.5 µM V1/V2 or F1/F2 was used for species-specific PCR 2.	94° for 3′; then 19 cycles of: 94° for 30″, annealing at 58° for 90″, extension at 72° for 90″; and finally 72° for 7′. 34 cycles were performed for species-specific subsequent PCR2, as above.
MSP3-alpha nested PCR (PCR 4 and 5)		
Primer sequence* (forward/reverse)	PCR-Mix†	PCR-Cycling‡
PCR 4: M1/M2: CAGCAGACACCATTTAAGG/ CCGTTTGTTGATTAGTTGC PCR 5: N1/N2: GACCAGTGTGATACCATTAACC/ ATACTGGTTCTTCGTCTTCAGG	PCR3: 2.5 mM $MgCl_2$, 150 µM dNTP, 0.15 µM M1/M2 or N1/N2, 0.5 U Taq-p, 3µL DNA. PCR4: Idem but 1µL DNA product of PCR3.	94° for 3′; then 34 cycles of: 94° for 30″, annealing at 50° for 30″, extension at 68° for 150″; and finally 68° for 4′.
Tandem repeat PCR (PCR 6 to 14)		
Primer Sequence* (forward/Reverse)	PCR-Mix†	PCR-Cycling‡
MN3: GAGAGTGACTAGCAATGG/ AGATGCCTTTTTCTGCGTTT MN4: TCACTTGGTTCTTCCCCG/ AGTGTGAACATGGGTGCA MN12: TCTGTTTCTGTTTCTGCGTT/ AGGGTTGTTTAACATCTGCT MN17: AACCGACTGGCTTACAACCA/ GATGTGGACGTCTGCATAGT MN23: AGCACAAGGTGACTCAAAAA/ AGCTGCGTTTTGATGGAGAA MN24: AATTTGACCTCCGGCACTTC/ TTCGTCACTTCGTCGTTTCG MN25: GGGGAAAAACAATGGCAA/ AGGCAGTCGATTTGAAACT MN26: TGAGCTGCGTAGAAGCCGTT/ TTGTTCAGCATGTTGCGTTG MN29: TGAAAAGAGCTGCTCGAACA/ CACTTGTAGAGAGAGGCGAG	2 mM $MgCl_2$, 150 µM dNTPs, 0.25 µM of each one of the pair of primers for the nine TR primer pairs (MN3, MN4, MN12, MN17, MN23, MN24, MN25, MN26, MN29), 0.5 U Taq-p, 3 µL DNA.	A single cycling protocol for all TR-PCR was used: 94°C for 2′; 35 cycles of: 94°C for 20″, 55°C for 10″, 65°C for 2′, and an extension step of 65°C for 5′.

*Sequences are described 5′ to 3′.
†All PCR mixes had 0.25 µL at final volume.
‡PCR reactions were performed using a PTC100, MJ Research Inc. thermal cycler (Bio-Rad).
§Taq-p: Taq polymerase (Invitrogen).

Table 3.2. Baseline characteristics of study subjects: total enrollees, enrollees who developed one episode, and enrollees who developed > 1 episode, by study sites (proportion of each cell per row are in italics)*

Variable	Total (*N* = 1507)		1 infection (*N* = 1153)		> 1 infection (*N* = 354)		*P* value
	N	%	*N*	%	*N*	%	
Health Post (*N* = 1507)							< 0.0001
San Jose de Lupuna (SJL)	198	(13.1)	150	(13.3) *(75.8)*	48	(12.8) *(24.2)*	
Santo Tomas (STO)	575	(38.2)	446	(38.5) *(77.6)*	129	(37.1) *(22.4)*	
Mazan (MZ)	348	(23.1)	215	(18.6) *(61.8)*	133	(37.7) *(38.2)*	
Padrecocha (PAD)	386	(25.6)	342	(29.6) *(88.6)*	44	(12.5) *(11.4)*	
Health Post (*N* = 1507)							< 0.0001
STO, SJL, and PAD	1,159	(76.9)	938	(81.4) *(80.9)*	221	(62.3) *(19.1)*	
Mazan	348	(23.1)	215	(18.6) *(61.8)*	133	(37.7) *(38.2)*	
Time living in community (years) (*N* = 1507)							0.2143
Median (IQR)	11.0	(4–22)	12.0	(4–22)	10.0	(4–20)	
Age (years) (*N* = 1507)							0.6573
Mean (SD)	27.1	(17.0)	27.0	(17.2)	27.4	(16.2)	
							0.1568
≤ 4	46	(3.1)	33	(2.9) *(71.7)*	13	(3.7) *(28.3)*	
5–14	373	(24.8)	299	(25.9) *(80.2)*	74	(20.9) *(19.8)*	
15–44	844	(56.1)	630	(54.6) *(74.6)*	214	(60.5) *(25.4)*	
≥ 45	244	(16.2)	191	(16.6) *(78.3)*	53	(14.9) *(21.7)*	
Gender (*N* = 1507)							0.1653
Male	831	(55.1)	624	(54.1) *(75.1)*	207	(58.5) *(24.9)*	
Female	676	(44.9)	529	(45.9) *(78.3)*	147	(41.5) *(21.7)*	
Education (in years) (*N* = 1477)							0.8068
Illiterate or < 6 years of age	53	(3.6)	39	(3.4) *(73.6)*	14	(4.1) *(26.4)*	
0–6	748	(50.6)	582	(51.1) *(77.8)*	166	(49.0) *(22.2)*	
7–11	632	(42.8)	482	(42.4) *(76.3)*	150	(44.2) *(23.7)*	
12–19	44	(3.0)	35	(3.1) *(79.5)*	9	(2.7) *(20.5)*	
Job (*N* = 1490)							< 0.0001
Requires travel out of village	286	(19.2)	188	(16.5) *(65.7)*	98	(28.0) *(34.3)*	
Does not require travel out of village	1204	(80.8)	952	(83.5) *(79.1)*	252	(72.0) *(20.9)*	
Total number of malaria cases in lifetime (*N* = 1507)							< 0.0001
Median (IQR)	3.0	(2–6)	3.0	(1—5)	4.0	(2–8)	
Traveled before any of the last 4 episodes (*N* = 1446)							< 0.0001
Yes	349	(24.1)	216	(19.8) *(61.9)*	133	(37.6) *(38,1)*	
No	1097	(75.9)	876	(80.2) *(79.9)*	221	(62.4) *(20.1)*	
Travel in past month (*N* = 1489)				< 0.0001			
No	1305	(87.6)	1032	(90.5)	273	(78.5)	
Yes	184	(12.4)	109	(9.5)	75	(21.5)	
Of those who traveled past month, number of trips (*N* = 184)							0.4623
Median (IQR)	1,0	(1–1.5)	1.0	(1–1)	1.0	(1–2)	
Traveled > 10 km, > 3days, not to Iquitos in the past month (*N* = 1490)							< 0.0001
Yes	159	(10.7)	95	(8.3) *(59.7)*	64	(18.3) *(40.3)*	
No	1331	(89.3)	1046	(91.7) *(78.6)*	285	(81.7) *(21.4)*	

*IQR = interquartile range.

Table 3.3. MSP3α-HhaI PCR-restriction fragment length polymorphism alleles.

Hha I								
Pattern	Allele							
1	Allele 1	1070	431	275	207			
2	Allele 2	1070	530	275	236			
3	Allele 3	1070	275	223				
4	Allele 4	1070	500	431				
5	Complex	1070	530	431	275	223		
6	Allele 5	1070	223					
7	Allele 6	1070	431	275	223			
8	Allele 7	1070	385	275	188			
9	Allele 8	1070	475	275	204			
10	Allele 9	1070	343	275	223			
11	Complex	1070	500	431	275	236		
12	Complex	1070	603	501	426	275	223	
13	Allele 10	1070	510	275	226			
14	Complex	1070	460	431	275	188		
15	Complex	1070	500	475	431	343	275	223

Table 3.4. MSP3α-AluI PCR-restriction fragment length polymorphism alleles.

Alu I								
Pattern	Allele							
1	Allele 1	551	412	354	259	205	153	
2	Allele 2	551	467	259	185	153		
3	Allele 3	551	398	349	259	192	173	153
4	Allele 4	551	205	153				
5	Allele 5	551	441	259	205	180	153	
6	Complex	551	464	412	293	259	192	153
7	Allele 6	551	447	205	175	153		
8	Allele 7	551	405	205	175	153		
9	Allele 8	551	398	259	205	175	144	
10	Complex	551	441	354	293	259	205	153
11	Allele 11	440	354	293	165	153		
12	Allele 12	551	354	259	205	173	153	
13	Allele 13	523	253	198	144	130		
14	Allele 14	500	398	205	175	153		

Table 3.5. Schematic table for all nine tandem repeat markers used, allelic types found in the study, and gene diversity (heterozygosity)*

Locus	MN3	MN4	MN12	MN17	MN23	MN24	MN25	MN26	MN29
Alleles at each locus	167	134	210	242	227	125	151	169	143
	212	143	229	258	244	143†	168	215	164
	240†	152	244†	275†	265†	160	180	230	175
	270	162†*		300	286		196	250	194†
	300	172			310		220	272	205
		186			330		239	300	217
		206			350		256	330†	235
					383		281†	360	275
							300	400	
							330	430	
%‡	57	41	34	51	26	47	21	21	42
He§	*0.54*	*0.70*	*0.73*	*0.60*	*0.87*	*0.58*	*0.88*	*0.88*	*0.73*

*Alleles were considered the same if molecular weights were within 5% of the band size.

†Indicates the most common allele for that locus.

‡Frequency of most common allele for that locus.

§Genetic diversity was assessed by measuring expected heterozygosity (*He*: chance of drawing 2 different alleles from a population sample) by use of the formula $H = n/(n\text{-}1)(1\text{-}\Sigma p_i^2)$, where p is the frequency of the ith allele and n is the sample size.

Table 3.6. Comparison between relapse and reinfection subjects*

Variable	Total (N = 336)		Relapse (N = 92)		Reinfection (N = 244)		P value
	N	%	N	%	N	%	
Health Post (N = 336)							0.2209
SJL	43	(12.8)	12	(13.0)	31	(12.7)	
STO	126	(37.5)	28	(30.4)	98	(40.2)	
Mazan	132	(39.3)	44	(47.8)	88	(39.3)	
PAD	35	(10.4)	8	(8.7)	27	(10.4)	
Health Post (N = 336)							0.0490
STO, SJL, and PAD	204	(60.7)	48	(52.2)	156	(63.9)	
Mazan	132	(39.3)	44	(47.8)	88	(36.1)	
Time living in community (years) (N = 353)							0.2014
Median (IQR)	10.0	(4–20)	9.0	(3–18)	10.0	(4–20)	
Age (years) (N = 352)							0.2301
Mean (SD)	27.4	(16.2)	29.0	(17.3)	26.6	(15.6)	
							0.3264
≤ 4	13	(3.9)	5	(5.4)	8	(3.3)	
5–14	72	(21.4)	15	(16.3)	57	(23.4)	
15–44	201	(59.8)	55	(59.8)	146	(59.8)	
≥ 45	50	(14.9)	17	(18.5)	33	(13.5)	
Gender (N = 336)							0.5181
Male	195	(58.0)	56	(60.9)	139	(57.0)	
Female	141	(42.0)	36	(39.1)	105	(43.0)	
Education (in years) (N = 322)							0.1343
Illiterate or < 6 years old	14	(4.4)	6	(6.9)	8	(3.4)	
0–6	159	(49.4)	47	(54.0)	112	(47.7)	
7–11	142	(44.1)	34	(39.1)	108	(45.7)	
12–19	7	(2.2)	0	(0.0)	7	(2.3)	
Job (N = 333)							0.3877
Requires travel out of village	91	(27.3)	22	(23.9)	69	(28.6)	
Does not require travel out of village	242	(72.7)	70	(76.1)	172	(71.4)	
Total number of malaria cases in lifetime (N = 353)							0.3716
Median (IQR)	4.0	(2–8)	4.0	(2–6.5)	4.0	(2–8)	
Traveled prior to any of the last 4 episodes (N = 336)							0.5004
Yes	129	(38.4)	38	(41.3)	91	(37.3)	
No	207	(61.6)	54	(58.7)	153	(62.7)	
Traveled in past month (N = 332)							0.0272
No	258	(77.7)	64	(69.6)	194	(80.8)	
Yes	74	(22.3)	28	(30.4)	46	(19.2)	
Of those who traveled in past month, number of trips (N = 76)							0.3200
Median (IQR)	1.0	(1–2)	1.0	(1–2)	1.0	(1–1)	
Traveled > 10 km, > 3 days, not to Iquitos in the past month (N = 332)							0.3750
Yes	62	(18.7)	20	(21.7)	42	(17.5)	
No	270	(81.3)	72	(78.3)	198	(82.5)	

*SLJ = San Jose de Lupuna; STO = Santa Tomas; PAD = Padrecocha; IQR = interquartile range.

Table 3.7. Lifetime malaria cases for those enrolled with > 1 episode versus those enrolled with only 1 episode, stratified by age.

Age	(n) mean (IQR)*	OR†	95% CI	P
<5	(46) 2 (0–3)	1.035	0.803–1.335	0.7896
5–14	(346) 2.9 (1–4)	1.105	1.027–1.188	0.0072
15–44	(844) 4.58 (2–6)	1.046	1.013–1.080	0.0055
>45	(244) 7.14 (3–10)	1.019	0.970–1.070	0.4569

*The total number of lifetime malaria cases stratified by age.
†The odds of those enrolled with >1 episode having increased lifetime malaria cases compared with those who were enrolled with only one episode.

Table 3.8. Bivariate logistic regression modeling probability of > 1 infection, relapse episodes using msp3α, and relapse episodes using tandem repeats*.

	1 infection vs. > 1 infection		Reinfection vs. relapse (Msp3 α)		Reinfection vs. relapse (tandem repeat)	
Variable	OR	95% CI	OR	95% CI	OR	95% CI
Health Post						
STO	1.00		1.00		1.00	
SJL	0.927	0.636–1.350	0.980	0.443–2.167	2.236	0.574–8.705
Mazan	2.054	1.532–2.753	1.603	0.923–2.785	3.131	1.092–8.973
Padrecocha	0.427	0.295–1.350	0.950	0.389–2.319	1.171	0.219–6.265
Health Post						
STO, SJL, and PAD	1.00		1.00		1.00	
Mazan	2.640	2.033–3.429	1.625	1.00–2.640	2.418	1.062–5.506
Time living in community (years)	0.994	0.986–1.003	0.991	0.974–1.009	0.983	0.951–1.017
Age (years)	1.002	0.995–1.009	1.009	0.994–1.024	0.993	0.967–1.019
< 5	1.420	0.698–2.889	1.214	0.344–4.283	1.800	0.304–10.644
5–14	0.892	0.600–1.326	0.511	0.226–1.155	0.548	0.184–1.634
15–44	1.216	0.864–1.712	0.731	0.377–1.418	0.806	0.232–2.800
≥ 45	1.00		1.00		1.00	
Male Gender	1.186	0.932–1.510	1.175	0.720–1.917	0.989	0.395–2.041
Education (in years) (not enough subjects in 12–19)						
N/A Under 6 years old	1.154	0.610–2.182	2.382	0.772–7.349	6.129	1.372–27.377
0–6	0.917	0.713–1.179	1.333	0.797–2.230	1.959	0.768–4.995
7–11	1.00		1.00		1.00	
12–19	0.826	0.388–2.182	–	–	–	–
Job requires travel out of the village	1.969	1.487–2.608	0.783	0.450–1.364	0.977	0.394–2.418
Total number of malaria cases in lifetime	1.042	1.018–1.066	0.985	0.930–1.043	0.970	0.879–1.071
Traveled before any of the last 4 episodes	2.411	1.856–3.133	1.183	0.725–1.930	1.375	0.604–3.128
Times traveled in past month	1.635	1.337–1.998	1.461	1.064–2.004	1.230	0.690–2.193
Travel in past month	2.601	1.883–3.592	1.845	1.067–3.193	1.882	0.777–4.559
Of those who traveled in past month, times	1.042	0.730–1.488	1.402	0.813–2.417	0.433	0.067–2.787
Traveled > 10 km and > 3 days in past month	2.473	1.755–3.484	1.310	0.721–2.379	2.225	0.914–5.413
Household variables						
Exterior wall materials						
Brick/Cement	1.00		1.00		1.00	
Wood	1.121	0.761–1.653	2.231	0.935–5.324	1.202	0.334–4.329
Other	0.714	0.440–1.160	1.109	0.361–3.406	0.634	0.101–4.001
Interior wall materials						
Brick/Cement	1.00		1.00		1.00	
Wood	1.031	0.580–1.834	2.818	0.613–12.963	–	–
Other	0.722	0.401–1.302	2.257	0.476–10.704	–	–
Location of kitchen						
Exterior	1.00		1.00		1.00	
Interior	1.033	0.783–1.362	0.817	0.473–1.411	0.760	0.297–1.947
Roof material						
Calamine	1.00		1.00		1.00	
Palm tree	1.225	0.916–1.639	1.475	0.805–2.703	1.969	0.642–6.036
Number bed nets/per bed	0.998	0.777–1.282	1.758	0.943–3.277	1.642	0.995–2.710
Possessions						
Radio	0.814	0.614–1.078	0.654	0.367–1.164	0.082	0.011–0.622
Black and white TV	0.785	0.531–1.160	1.081	0.514–2.276	1.633	0.366–7.300
Color TV	1.240	0.887–1.732	1.070	0.544–2.104	1.507	0.428–5.311
VHS/DVD	1.191	0.700–2.026	0.743	0.269–2.056	1.404	0.178–11.093
Audio stereo	0.743	0.404–1.367	0.746	0.247–2.257	0.971	0.120–7.853

*STO = Santa Tomas; SJL = San Jose de Lupina; PAD = Padrecocha.

Table 3.9. Multivariate logistic regression modeling probability of > 1 infection during study period*.

Variable	OR	95% CI
Health Post		
STO, SJL, and PAD	1.00	
Mazan	2.559	1.866–3.510
Age		
< 5	1.683	0.749–3.780
5–14	1.488	0.949–2.333
15–44	1.489	1.031–2.152
≥ 45	1.00	
Male gender	0.891	0.679–1.168
Job requires travel out of the village	1.453	1.027–2.056
Total number of malaria cases lifetime	1.064	1.037–1.092
Travel in past month	1.464	1.001–2.140

*OR = odds ratio; CI = confidence interval; STO = Santa Tomas; SJL = San Jose de Lupuna; PAD = Padrecocha.

CHAPTER 4: Genome Scale Analysis of Human Antibody Responses in *Plasmodium vivax* Relapse vs. Reinfection: Implications for Immunity and Sero-Epidemiological Surveillance

ABSTRACT

Determining human exposure to malaria for purposes of surveillance and control is time-intensive, often insensitive, and expensive because it is conventionally based on detection of parasitemia by light microscopy, rapid diagnostic tests or nucleic acid amplification-based assays. In this study, genome-scale protein microarray analysis based on proteins identified by transcriptional profiling of asexual stage-expressed *P. vivax* proteins was used to identify antigens recognized by sera obtained from patients residing in an endemic region. Patterns of antibody responses affected by sequential symptomatic *P. vivax* episodes, whether relapse or reinfection, were determined. Of 106 subjects who presented with a symptomatic *P. vivax* malaria episode, prospective study over 24-months showed that 91 had 2 additional symptomatic *P. vivax* malaria episodes, 11 had 3 episodes, 3 had 4 episodes, and 1 had 5 episodes. *P. vivax*-reinfected subjects reacted more strongly to the entire set of differentially recognized *P. vivax* blood stage antigens than did relapsing subjects independent of number of malaria episodes. Differential reactivity between reinfected and relapsed subjects of individual *P. vivax* antigens was not observed. The most highly recognized *P.*

vivax proteins were Merozoite Surface Protein (MSP) 7, 8, and 10 (PVX_082650, PVX_097625, PVX_114145); hypothetical proteins (PVX_081830, PVX_115450, PVX_117680, PVX_110935, PVX_097730, PVX_118705); and sexual stage antigen s16 (PVX_000930). Genes encoding differentially reactive proteins exhibited a significant enrichment of non-synonymous nucleotide variation, an observation consistent with immune selection. These results indicate that *P. vivax* reinfection boosts overall antibody responses against *P. vivax* asexual blood stage antigens more than *P. vivax* relapse. Further, this work identifies a large set of antigens that have undergone immune selection and are candidate sero-epidemiological tools that can be tested in more effective malaria control and elimination efforts in *P. vivax*-endemic regions.

INTRODUCTION

Current estimates of the global prevalence of *Plasmodium vivax* infection are based on identifying potential regions of transmission using a geo-referenced parasite-prevalence rate combined with biological masking to indicate malaria-compatible transmission zones.[1] Quantifying the global burden of *P. vivax* malaria is complicated by the biology of the parasite: *P. vivax* relapse from dormant hypnozoite liver stage parasites occurs over time and space—often asymptomatically—which allows for human migration to disperse infection within an endemic region or reintroduce the parasite into areas where malaria may have been eliminated. The potential range of *Plasmodium vivax* transmission is vast, the biology of the parasite complicates incidence and prevalence estimates, and the public health impact of *P.* vivax remains. In an era in which global malaria elimination and eradication are being contemplated,[2-4] it is essential to have accurate tools to estimate parasite prevalence, and malaria disease incidence.

Because determining the presence of malaria parasitemia or exposure is time-intensive, often insensitive, and expensive, interest has grown for using serological tools to monitor infection status and transmission dynamics—so called sero-epidemiology. This approach has been particularly useful for malaria as well as vector-borne diseases such as lymphatic filariasis in which mass drug administration campaigns have been carried out. In the past, immunofluorescence and ELISA using lysates of *in vitro* grown *P. falciparum*

schizonts have been used as antigen for antibody detection.[5-11] More recently, recombinant proteins based on vaccine candidates (Circumsporozoite Protein, MSP1, and AMA-1) have been the most common used tools for *P. falciparum* seroepidemiology studies. By analogy, for sero-epidemiological studies of *P. vivax*, recombinant PvMSP1, and PvAMA-1 have been used to determine seroprevalence of antibodies to *P. vivax* in populations of Vanuatu, Solomon Islands.[12] More recently, a proof-of-principle array study using 152 predicted asexual stage proteins was reported in which sera from Korean *P. vivax* malaria patients were analyzed for anti-*P. vivax* antibodies using a wheat germ expression system.[13] Because *P. vivax* is only available in relatively low parasitemia from humans and in limited quantities from non-human primates, sero-epidemiology studies using *P. vivax* asexual stage parasite lysates has only infrequently been done, hence recombinant proteins are essential for studying exposure to *P. vivax* in endemic populations.

To understand the *P. vivax* antigens predominantly recognized by the antibody response of naturally infected humans in a low-transmission region, we analyzed the serological reactivity of patients with *P. vivax* malaria using custom-made, genome-level protein microarrays comprised of asexual blood stage antigens predicted by transcriptional profiling[14,15] and heterologously produced by a prokaryotic cell-free expression system.[16-18] With relapses and reinfections being common in the study population (as defined using molecular tools), we were able to compare the level of IgG responses between

consecutive episodes of symptomatic *P. vivax* malaria, with the goal of determining if naturally acquired antibody responses were boosted by subsequent infection. The overall goal of these studies was to systematically and comprehensively identify *P. vivax* antigens of potential use for sero-epidemiological studies.

MATERIALS AND METHODS

Human subjects approvals. All patients provided written informed consent to be enrolled in this study, which was approved by the following institutional review boards: Ethical Committee of Universidad Peruana Cayetano Heredia, Lima, Peru; Ethical Committee of Asociación Benéfica PRISMA, Lima, Peru; the Directorate of Health of Loreto-Peru; the Humans Research Protection Program of the University of California at San Diego; and the Institutional Review Board of the Johns Hopkins Bloomberg School of Public Health.

Study sites. The field activities of this study were carried out from May 2005 to July 2008 in northeastern Peru, in the area near to the city of Iquitos in the province of Maynas, the capital of the Amazon Department of Loreto. Considering the region's geographical isolation from the rest of Peru, health services within the surrounding areas of Iquitos are relatively good and accessible. Three health posts provide medical services to the study villages described as follows. Santo Tomas health post, located 16 km from Iquitos by road and surrounded by the Nanay River, is a referral health center for 3 villages: La Unión, 12 de Diciembre, and Santo Tomás, with a catchment area of 2,650 inhabitants. San Jose de Lupuna health post, located 10 km from Iquitos is accessible only through the Nanay River, and is the referral center for 4 villages: San Pedro, Santa Rita, Fray Martín, and San Jose de Lupuna with a catchment area of 1,250 inhabitants. Padrecocha health post, located 6 km from Iquitos is accessible only through the Nanay River and is a referral

health post for 3 villages, San Andrés, Nueva Vida, and Padrecocha, with a catchment area of 1800 inhabitants.

Blood sampling. Blood samples were collected from patients diagnosed by conventional light microscopy on site at Peruvian Ministry of Health establishments according to national norms. Patients identified as infected with only *P. vivax* (microscopy only) were invited to participate in the study. At each enrollment site, venous blood was collected in EDTA Vacutainer tubes (Becton Dickinson) and taken to the field project laboratory where samples were aliquoted and frozen at -20°C. Samples were shipped on dry ice to Universidad Peruana Cayetano Heredia in Lima for molecular analysis.

Molecular confirmation of P. vivax infection. DNA was extracted from 200 µL of thawed anticoagulated whole blood using the Qiagen Blood Kit (Qiagen, Valencia, CA). The diagnosis of *P. vivax* infections was confirmed in all patients using a genus and species-specific nested PCR assay; samples containing *P. falciparum* or mixed infections were not considered for further analysis*, P. malariae* or *P. ovale* were not found. A nested PCR was used based on an 18S ribosomal RNA gene fragment specific for the genus *Plasmodium*. A 1200 base pair (bp) fragment allowed for *Plasmodium* genus-level identification; identification of *P. vivax* and *P. falciparum* species was done using a 120 bp fragment specific for *P. vivax* and a 205 bp fragment specific for *P. falciparum*.

Molecular genotyping. Genotyping of *P. vivax* isolates was performed using PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of the *P. vivax merozoite surface protein-3alpha* (*PvMSP3α*) gene as previously described.[19]

Case definition and data analysis. Relapse was defined as identical *PvMSP3-α* PCR-RFLP patterns when primary and subsequent infection parasites were compared. Reinfection was defined as different genotypes present in primary vs. subsequent infection parasites by *PvMSP3-α* PCR-RFLP patterns.

Protein microarray analysis using a P. vivax asexual blood stage proteome array. A proteome array was designed and fabricated containing 2,233 *P. vivax* recombinant proteins, representing 1,936 predicted *P. vivax* asexual blood stage proteins. Proteins predicted to be secreted or present on the parasite surface, on the basis of the presence of a secretory signal peptide or transmembrane domains, were annotated as such in *PlasmoDB*[20,21] and preferentially included. Additional selection for expression of *P. vivax* genes included expression microarray evidence for blood stage expression.[14,15] Putative cytoplasmic proteins, lacking both signal peptides and transmembrane domains, were also selected (pI < 7.6 for *P. vivax* genes). *P. vivax vir* genes were excluded because of their variability. Both single and multi-exon genes were selected. Exons of multi-exon genes were cloned separately. Furthermore, large genes or exons were further divided into

overlapping segments to limit amplicon length between 300 and 3000 nt. Amplicons were labeled with the exon number and the total number of exons, such as “1o2” for exon 1 of a 2-exon gene. Genes that were further divided into segments were labeled s1, s2, etc.

The array was fabricated as previously described.[22] Briefly, amplified genes from *P. vivax* genomic DNA (Sal I strain [MRA-552, MR4]) were cloned into the PXT7 plasmid with a T7 transcription terminator and encoded 5’ polyhistidine (HIS) and 3’ hemagglutinin (HA) epitopes. Recombinant proteins were expressed by an *E. coli* cell-free *in vitro* transcription and translation system (RTS 100 HY kits from 5 PRIME, Gaithersburg, MD) according to the manufacturer’s instructions. Proteome arrays were printed as previously described, with each recombinant protein spotted once in each array.[22] Each array contained 256 negative control spots made with an *in vitro* transcription translation master mix without plasmid DNA. Once printed, recombinant protein expression was verified using anti-polyhistidine (clone His-1, Sigma) and anti-hemagglutinin (clone 3F10, Roche) monoclonal antibodies, as previously described.[22] All signal intensities were corrected for spot-specific background. A recombinant protein was deemed to be present on the slide if its spot’s mean fluorescence intensity was that of the mean of the “no DNA” control spots plus two standard deviations.

Overall, 1,470 different *P. vivax* recombinant proteins (66%) were expressed with both the His and HA tags, confirming that the majority of

recombinant proteins were fully expressed. These represented 1,328 *P. vivax* predicted asexual blood stage proteins. Furthermore 1,949 *P. vivax* recombinant proteins (87%) were expressed with at least one tag suggesting that these recombinant proteins were at least partially expressed.

Fluorescence was detected and analyzed using Perkin Elmer ScanArray Express HT microarray scanner. All signal intensities were corrected for spot-specific background, the signal from the substrate surrounding the spots. The expression of the polypeptides was determined based on a cutoff of the mean signal of the “no DNA” control spots plus two standard deviations.

Microarray Data Analysis. Signal values from microarray scans were normalized by the vsn method and a Bayes T test was used to identify differentially reactive antigens. Statistical analysis was performed in the R environment (www.r-project.org). Data was transformed by variance stabilization using the vsn method from the Bioconductor suite (www.bioconductor.org) using spotted IgG positive controls as normalization controls. Negative control was based on the product of empty vector transcription/translation reactions; these mean signal intensities were subtracted from protein detection signal intensities. Bayes regularized t-test adapted from Cyber-T for protein arrays[23] was employed to compare the reactivity to the antigens between the relapse and reinfection groups; the Bonferroni method was used to correct for multiple measurements, estimate

the False Discovery Rate (FDR), and provide corrected *p* values. Proteins with transformed signal values >2 standard deviations over the mean number of negative control (empty vector products spotted onto array) signal values were considered as reactive.

Analysis of genome-wide P. vivax single nucleotide polymorphisms. The genes encoding were compared to a globally represented set of annotated *P. vivax* genomes (parasites from Brazil, India, Mauritania and North Korea), available at https://www.broadinstitute.org/annotation/genome/plasmodium_vivax/GenomeDescriptions.html (accessed May 10, 2014).

RESULTS

Number of *Plasmodium vivax* malaria episodes. In the study period, 106 subjects were enrolled who had 1-5 episodes of symptomatic light microscope- and PCR-confirmed *P. vivax* malaria (Table 4.1)(Fig. 4.1). The median age was 24 years old, main age was 27 years old, and 48% of the subjects were women. Occupation and demographics of the study participants were typical of the region (Table 4.2).

All patients were given standard antimalarial treatment following guidelines from the Peruvian Ministry of Health (chloroquine for 3 days (10mg/kg on days 1 and 2; and 5mg/kg on day 3), plus primaquine (0.5 mg/kg/day for 7 days)). Of the 106 subjects, 91 had 2 episodes, 11 had 3 episodes, 3 had 4 episodes, and 1 had 5 episodes. Using MSP 3-alpha genotyping, of the recurrent infection episodes, 48 had identical genotypes compared to a previous infection leading to classification as relapse; 78 had different genotypes and were classified as reinfection. This classification is limited by the possibility of a subject harboring more than one *P. vivax* clone and hence overestimates the rate of reinfection compared to relapse. Subjects with *P. vivax* malaria tended to report more lifetime episodes of malaria than the episodes observed during the study period (Table 4.1). The average number of self-described malaria lifetime episodes for all participants was 4.8; for relapsing subjects, 4.3 episodes, and for reinfection subjects, 5.2.

Descriptive Analysis of serological reactivity to *Plasmodium vivax* antigens using a protein microarray. Of the 2233 *P.* vivax protein spots (derived from 1470 genes) on the protein microarray (see Supplementary Table 1 for complete list of arrayed recombinant proteins), a total of 280 proteins were differentially reactive compared to a negative control group (N=18, USA residents with no history of malaria), after correcting for multiple measurements using the Bonferroni test. The most differentially reactive antigens were Merozoite Surface Proteins (MSP) 7, 8 and 10, several hypothetical proteins and the putative sexual stage antigen s16. The top 10 differentially reactive proteins and gene for all *P. vivax*-infected subjects are listed in Table 4.3. The complete list of differentially reactive proteins is presented in Supplementary Table 2.

Differentially recognized *Plasmodium vivax* antigens occurred more commonly in reinfection than in relapse and reinfection had higher cumulative IgG signal than relapse. *P. vivax* parasitemia may reflect one of several clinical states: relapse (which may be symptomatic or, most often in the Peruvian Amazon region, asymptomatic new infection,[24] or a continuing previously un- or inadequately treated blood stage infection. Subjects studied here represent either relapse or reinfection because blood stage *P. vivax* is not known to be resistant to chloroquine in the region and all patients were treated with directly observed therapy (DOT) with standard therapy (chloroquine and primaquine). In this study, reinfection was defined as

different genotypes present in primary vs. subsequent infection parasites by *PvMSP3-α* PCR-RFLP patterns.

The antigens differentially recognized by *P. vivax*-infected subjects were more likely to have significantly higher cumulative signal for reinfection subjects than for relapse (Fig. 4.3). In the present study, a new *P. vivax* infection was only determined for symptomatic subjects.

Operationally, subjects who develop a recurrent infection within 28 days after prior episode are considered as treatment failure or recrudescence and could potentially be due to tolerance or resistance to treatment. In the present study, no one develop a recurrent episode within 28 days after prior treatment. One subject developed a recurrent episode after 29 days but it had a different *PvMSP3-α* haplotype, therefore it was a reinfection.

Enrichment of genetic diversity of genes encoding differentially reactive antigens. The non-synonymous genetic diversity (π) of genes encoding the set of differentially-recognized *P. vivax* antigens was compared with coding sequences found in the *P. vivax* genome as a whole. This analysis, using globally representative *P. vivax* strains from Brazil, India, Mauritania and North Korea, only included annotated genes; Vir family members, hypothetical genes were not included. The set of genes encoding the differentially-recognized *P. vivax* antigens as a whole (combining proteins differentially recognized by both relapse and reinfection patients) had a significantly higher level of single nucleotide polymorphism (SNP) diversity than the rest of the coding genes, as

assessed by the Mann Whitney test (W = 249177, *p*-value = 0.0007472). Genes encoding the set of differentially recognized proteins were significantly more likely to have 1 or more non-synonymous SNPs (W = 249791, *p*-value = 0.0006419). In contrast, the level of synonymous SNPs in genes encoding differentially recognized proteins was not significantly different (W = 291635, *p*-value = 0.3948). This analysis suggests that positive immune selection for amino acid polymorphism in these *P. vivax* antigens has taken place.

DISCUSSION

Infections by malaria parasites are known to lead to broadly reactive anti-*Plasmodium* antibody responses.[25] How to analyze large-scale antibody responses in key areas of malaria research such as delineating target antigens of protective immunity or how to identify new proteins for sero-epidemiological analyses of malaria transmission in endemic regions are important applications of current systems immunology methodologies.[18] In the present study, sera from well-characterized symptomatically relapsed or reinfected *P. vivax*-infected subjects in the low-transmission region of the Peruvian Amazon were used to probe genome-scale *P. vivax* protein microarrays. These experiments produced several key conclusions: 1) that a relatively small set of differentially reactive *P. vivax* antigens are sufficient to identify *P. vivax* infection whether due to relapse or reinfection; 2) that reinfection is associated with higher cumulative intensity signal on the protein arrays against the complete set of reactive antigens than relapsed infection; and 3) that the differentially reactive antigens have undergone immune selection as reflected by the statistically more common non-synonymous nucleotide changes in the detected *P. vivax* antigens in response to host-parasite interactions.

The cumulative total antibody response is greater in reinfection compared to relapsed infection, which has implications for understanding immunological mechanisms and consequences of relapsing vivax malaria.

Previous studies have suggested that asymptomatic relapse is common in *P. vivax* malaria,[24] despite the more widely recognized phenomenon of relapse leading to symptomatic infection and the seeking of medical care. The present investigation provides evidence that reinfection resulting in clinical symptoms is associated with a more vigorous antibody response than is relapsing symptomatic infection, raising the possibility that there is a partially effective immune response to a previously experienced *P. vivax* strain resulting in less of an elevation of antibody response. Alternatively, it is also possible that lower parasitemia in relapse compared to reinfection might be associated with a lower antibody response, but the present data are not consistent with this possibility because the parasitemias were only semi-quantitatively assessed (1-4 + system) in this study.

The present protein microarray analysis provides immediate tools to apply to sero-epidemiology of malaria with regard to surveillance, control and elimination. Future work will be to establishing the population decay kinetics of antibody responses to different specific antigens that will allow investigators to infer an approximate time of previous infection. Such studies are essential because previous seroepidemiological studies have relied on blood stage vaccine candidate antigens selected for high-titered, immunodominant and sustained antibody responses, rather than with regard to differential seroreactivity in human populations that would diminish over time. The use of parasite lysates for ELISA or immunofluorescent microscopy is not applicable

to such studies of decay kinetics because of their heterogeneity and low sensitivity/specificity for inferring previous time of infection. The ideal antigen(s) for determining recent vs. remote malaria transmission from mosquitoes to residents of endemic areas would have the properties of being associated with rapid vs. sustained decay kinetics.

The *P. vivax* antigen yielding the most consistently high intensity reactivity on the protein array was PvMSP10; others top ranked antigens included MSP7, MSP8 and several hypothetical proteins, and a putatively sexual stage antigen s16. Antibodies to PvMSP10 have previously been readily detected in humans with *P. vivax* malaria[26,27] using *E. coli*-[26] and wheat germ *in vitro* expression system-expressed protein.[27] Studies with *P. falciparum* MSP10 demonstrated a lack of cross-reactivity with other merozoite surface proteins (PfMSP-1, -4, -5 and -8), consistent with the 95% specificity for *P. vivax* infection found for antibodies detected against wheat germ-expressed PvMSP10.[27] No experimental evidence has emerged to date regarding the viability of any *Plasmodium* spp. MSP10 as a candidate for an asexual blood stage-based vaccine.

ACKNOWLEDGMENTS

I would like to acknowledge co-authors Douglas Molina, Huw Davies, Eli Moss, Ruobing Wang, Malcolm Gardner, Kimberly Brouwer, Sonia Torres, Robert Gilman, Hugo Rodriguez, Alejandro Llanos, Daniel Neafsey, and Philip Felgner, for their contribution to this paper and permission to use it as part of my dissertation. I also thank Paula Maguina for expert and essential scientific, ethics and logistical contributions to allowing this work to come to fruition

This work was supported by U.S. Public Health Service grants from the National Institutes of Health: U19AI089681, R01AI067727, K24AI068903, D43TW007120 (JV) R01AI05759206, SBIR grant AI075692, and R01RHLO86488.

REFERENCES

1. Gething PW, Elyazar IR, Moyes CL, Smith DL, Battle KE, Guerra CA, Patil AP, Tatem AJ, Howes RE, Myers MF, et al: A long neglected world malaria map: Plasmodium vivax endemicity in 2010. *PLoS Negl Trop Dis* 2012, 6:e1814.

2. Cotter C, Sturrock HJ, Hsiang MS, Liu J, Phillips AA, Hwang J, Gueye CS, Fullman N, Gosling RD, Feachem RG: The changing epidemiology of malaria elimination: new strategies for new challenges. *Lancet* 2013, 382:900-911.

3. Feachem RG, Phillips AA, Hwang J, Cotter C, Wielgosz B, Greenwood BM, Sabot O, Rodriguez MH, Abeyasinghe RR, Ghebreyesus TA, Snow RW: Shrinking the malaria map: progress and prospects. *Lancet* 2010, 376:1566-1578.

4. White NJ, Pukrittayakamee S, Hien TT, Faiz MA, Mokuolu OA, Dondorp AM: Malaria. *Lancet* 2014, 383:723-735.

5. Arnold BF, Priest JW, Hamlin KL, Moss DM, Colford JM, Jr., Lammie PJ: Serological measures of malaria transmission in haiti: comparison of longitudinal and cross-sectional methods. *PLoS One* 2014, 9:e93684.

6. Cook J, Speybroeck N, Sochanta T, Somony H, Sokny M, Claes F, Lemmens K, Theisen M, Soares IS, D'Alessandro U, et al: Sero-epidemiological evaluation of changes in Plasmodium falciparum and Plasmodium vivax transmission patterns over the rainy season in Cambodia. *Malar J* 2012, 11:86.

7. De Carvalho ME, Ferreira MU, De Souza MR, Ninomia RT, Matos GF, Camargo LM, Ferreira CS: Malaria seroepidemiology: comparison between indirect fluorescent antibody test and enzyme immunoassay using bloodspot eluates. *Mem Inst Oswaldo Cruz* 1992, 87:205-208.

8. Fowkes FJ, Richards JS, Simpson JA, Beeson JG: The relationship between anti-merozoite antibodies and incidence of Plasmodium falciparum malaria: A systematic review and meta-analysis. *PLoS Med* 2010, 7:e1000218.

9. Stewart L, Gosling R, Griffin J, Gesase S, Campo J, Hashim R, Masika P, Mosha J, Bousema T, Shekalaghe S, et al: Rapid assessment of

malaria transmission using age-specific sero-conversion rates. *PLoS One* 2009, 4:e6083.

10. Warren M, Collins WE, Jeffery GM, Skinner JC: The seroepidemiology of malaria in Middle America. V. Antibody responses in an indicator population from an endemic area with attack phase antimalaria activities. *Am J Trop Med Hyg* 1983, 32:1209-1215.

11. Tongol-Rivera P, Kano S, Miguel E, Tongol P, Suzuki M: Application of seroepidemiology in identification of local foci in a malarious community in Palawan, The Philippines. *Am J Trop Med Hyg* 1993, 49:608-612.

12. Cook J, Reid H, Iavro J, Kuwahata M, Taleo G, Clements A, McCarthy J, Vallely A, Drakeley C: Using serological measures to monitor changes in malaria transmission in Vanuatu. *Malar J* 2010, 9:169.

13. Lu F, Li J, Wang B, Cheng Y, Kong DH, Cui L, Ha KS, Sattabongkot J, Tsuboi T, Han ET: Profiling the humoral immune responses to Plasmodium vivax infection and identification of candidate immunogenic rhoptry-associated membrane antigen (RAMA). *J Proteomics* 2014, 102C:66-82.

14. Bozdech Z, Mok S, Hu G, Imwong M, Jaidee A, Russell B, Ginsburg H, Nosten F, Day NP, White NJ, et al: The transcriptome of Plasmodium vivax reveals divergence and diversity of transcriptional regulation in malaria parasites. *Proc Natl Acad Sci U S A* 2008, 105:16290-16295.

15. Westenberger SJ, McClean CM, Chattopadhyay R, Dharia NV, Carlton JM, Barnwell JW, Collins WE, Hoffman SL, Zhou Y, Vinetz JM, Winzeler EA: A systems-based analysis of *Plasmodium vivax* lifecycle transcription from human to mosquito. *PLOS Neglected Tropical Diseases* 2010, in press.

16. Molina DM, Finney OC, Arevalo-Herrera M, Herrera S, Felgner PL, Gardner MJ, Liang X, Wang R: Plasmodium vivax pre-erythrocytic-stage antigen discovery: exploiting naturally acquired humoral responses. *Am J Trop Med Hyg* 2012, 87:460-469.

17. Doolan DL, Mu Y, Unal B, Sundaresh S, Hirst S, Valdez C, Randall A, Molina D, Liang X, Freilich DA, et al: Profiling humoral immune responses to P. falciparum infection with protein microarrays. *Proteomics* 2008, 8:4680-4694.

18. Crompton PD, Kayala MA, Traore B, Kayentao K, Ongoiba A, Weiss GE, Molina DM, Burk CR, Waisberg M, Jasinskas A, et al: A

prospective analysis of the Ab response to Plasmodium falciparum before and after a malaria season by protein microarray. *Proc Natl Acad Sci U S A* 2010, 107:6958-6963.

19. Cui L, Mascorro CN, Fan Q, Rzomp KA, Khuntirat B, Zhou G, Chen H, Yan G, Sattabongkot J: Genetic diversity and multiple infections of Plasmodium vivax malaria in Western Thailand. *Am J Trop Med Hyg* 2003, 68:613-619.

20. Aurrecoechea C, Brestelli J, Brunk BP, Dommer J, Fischer S, Gajria B, Gao X, Gingle A, Grant G, Harb OS, et al: PlasmoDB: a functional genomic database for malaria parasites. *Nucleic acids research* 2009, 37:D539-543.

21. Nielsen H, Brunak S, von Heijne G: Machine learning approaches for the prediction of signal peptides and other protein sorting signals. *Protein Eng* 1999, 12:3-9.

22. Molina D, Finney OC, Aravelo-Herrera M, Herrera S, Felgner PL, Gardner MJ, Liang X, Wang R: Plasmodium vivax pre-erythrocytic-stage antigen discovery: exploiting naturally acquired humoral responses. *Am J Trop Med Hyg* 2012:in press.

23. Baldi P, Long AD: A Bayesian framework for the analysis of microarray expression data: regularized t -test and statistical inferences of gene changes. *Bioinformatics* 2001, 17:509-519.

24. Van den Eede P, Soto-Calle VE, Delgado C, Gamboa D, Grande T, Rodriguez H, Llanos-Cuentas A, Anne J, D'Alessandro U, Erhart A: Plasmodium vivax sub-patent infections after radical treatment are common in Peruvian patients: results of a 1-year prospective cohort study. *PLoS One* 2011, 6:e16257.

25. Crompton PD, Moebius J, Portugal S, Waisberg M, Hart G, Garver LS, Miller LH, Barillas C, Pierce SK: Malaria immunity in man and mosquito: insights into unsolved mysteries of a deadly infectious disease. *Annu Rev Immunol* 2014, 32:157-187.

26. Perez-Leal O, Sierra AY, Barrero CA, Moncada C, Martinez P, Cortes J, Lopez Y, Salazar LM, Hoebeke J, Patarroyo MA: Identifying and characterising the Plasmodium falciparum merozoite surface protein 10 Plasmodium vivax homologue. *Biochem Biophys Res Commun* 2005, 331:1178-1184.

27. Cheng Y, Wang B, Sattabongkot J, Lim CS, Tsuboi T, Han ET: Immunogenicity and antigenicity of Plasmodium vivax merozoite surface protein 10. *Parasitol Res* 2014.

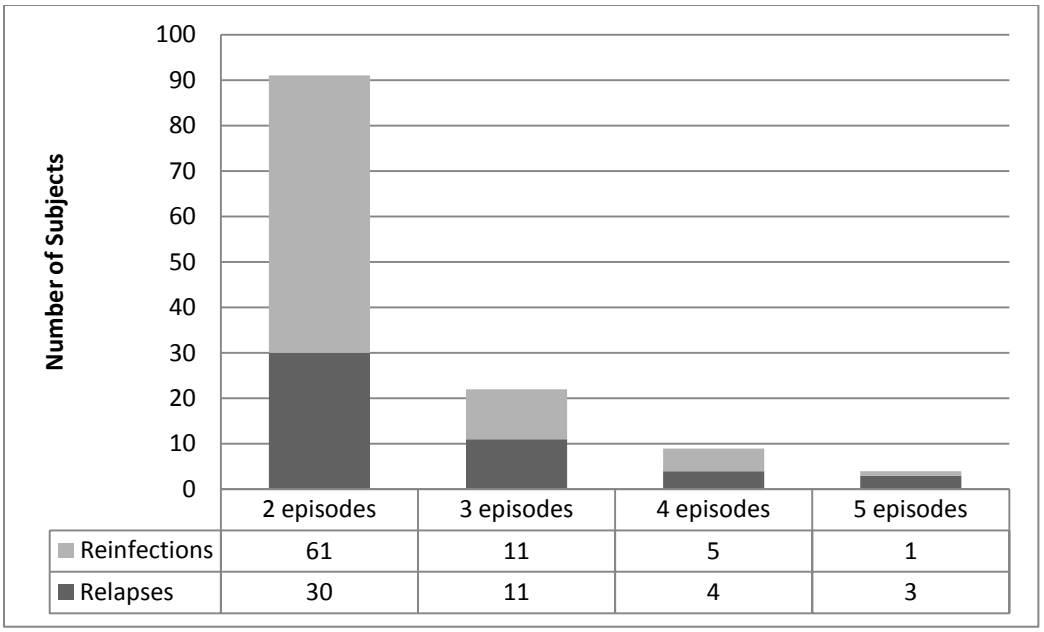

Figure 4.1. Number of *Plasmodium vivax* relapses and reinfections in patient population.

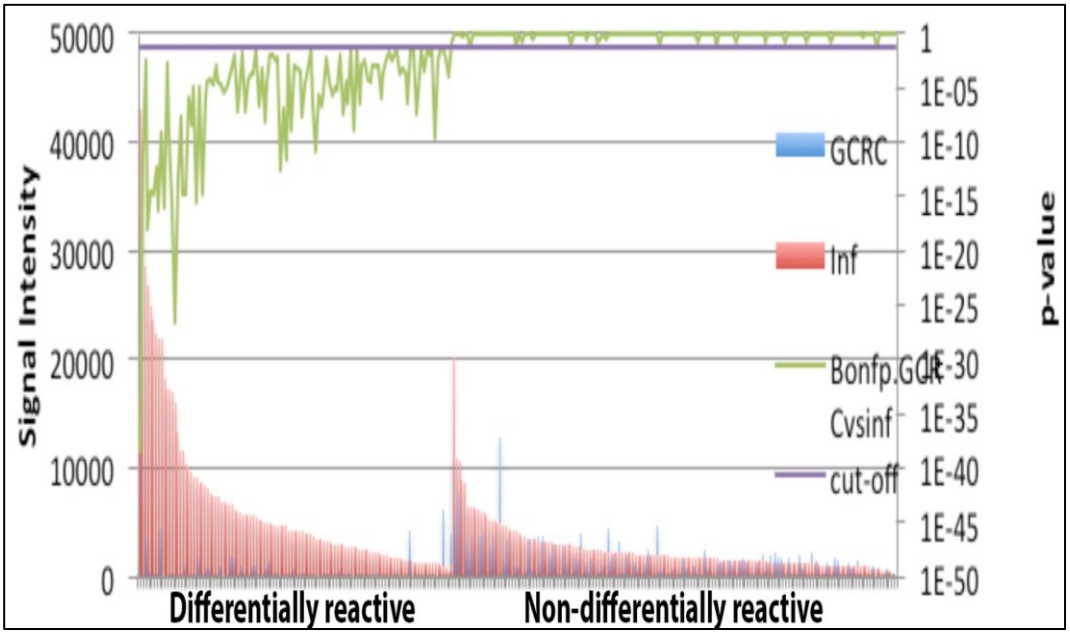

Figure 4.2. Protein microarray analysis of sera from first observed episode of *Plasmodium vivax* malaria. Differentially recognized proteins are indicated with a green line above them on the left side of the figure, where the *p*-value is less than 0.05 (corrected for multiple measures by the Bonferroni test). All *P. vivax*-infected (relapse plus reinfection) are indicated by (Inf) and negative controls are non-malaria infected individuals from the USA controls (GCRC, General Clinical Research Center). Because of space limitations, only representative proteins are shown along the x-axis; the full list can be found as Supplementary Table 1.

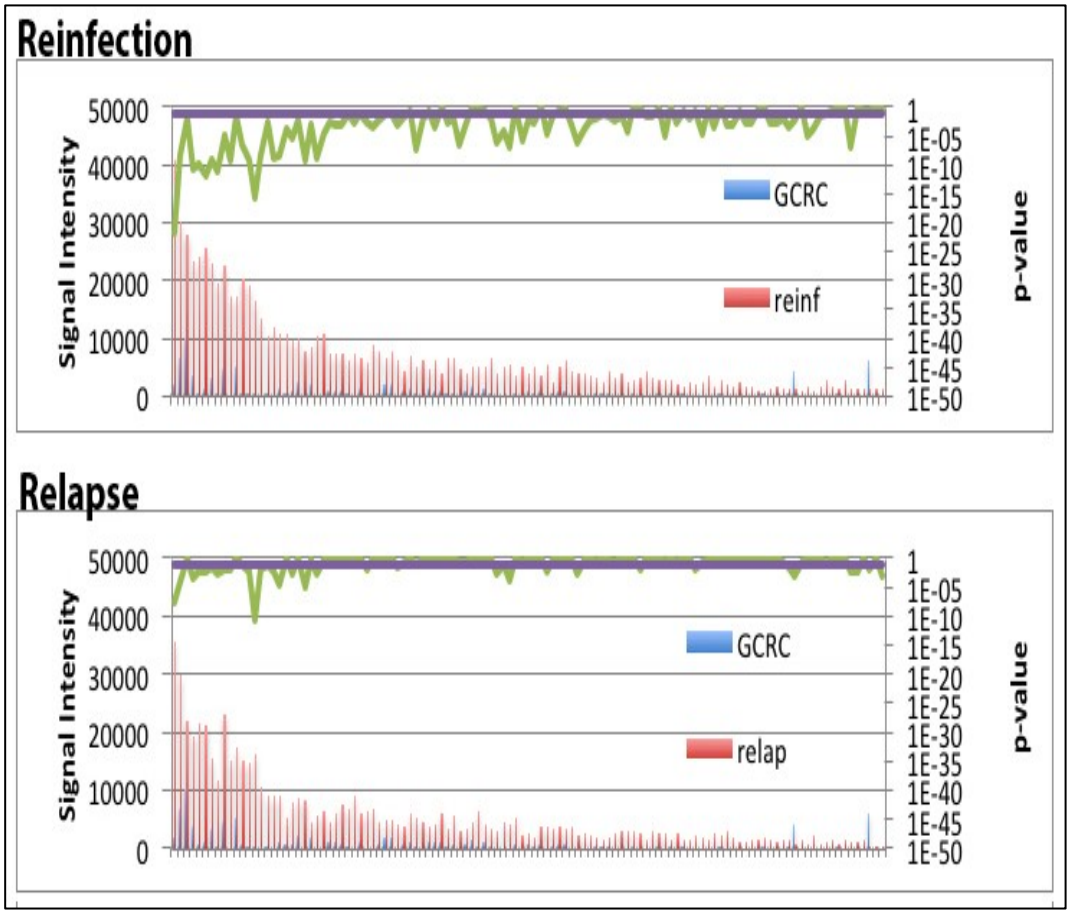

Figure 4.3. Protein microarray analysis comparing serological responses of subjects with *Plasmodium vivax* relapse to reinfection. The number of differentially reactive proteins and signal intensity of these proteins (top 10 proteins shown in Table 3) is significantly higher in the reinfection group compared to the relapse group (complete list in Supplementary Table 2). For clarity, proteins that are not differentially recognized are not shown.

Table 4.1. Characteristics of participants and recurrent episodes (relapses plus reinfections).

	Number	Mean age [Min - Max]	Gender	Median age	Mean time [Min-Max]	Mean lifetime malaria episodes
Subjects	106	27 [3 - 83] years	48% female	24 years	8.5 [1 - 24.2] months	4.8
Recurrences	126	[a]	[a]	[a]	8.5 [1 - 24.2] months	[a]
Relapses	48	26 [3 - 83] years	38% female	18 years	6 [1.2 - 20.4] months	4.3
Reinfections	78	28 [5 - 76] years	49% female	25 years	10 [1 - 24.2] months	5.2

[a] Same as shown for subjects above

Table 4.2. Occupation/demographic characteristics of *Plasmodium vivax* malaria subjects.

Occupation	Relapses (%)	Reinfections (%)	Total (%)
Student	23 (48)	25 (32)	48 (38)
Agriculture	7 (15)	12 (15)	19 (15)
Homemaker	6 (13)	20 (26)	26 (21)
Crafting	0 (0)	2 (3)	2 (2)
Transportation	1 (2)	3 (4)	4 (3)
Commerce	1 (2)	1 (1)	2 (2)
Construction	0 (0)	8 (10)	8 (6)
Unemployed	4 (8)	4 (5)	8 (6)
Fisherman	4 (8)	2 (3)	6 (5)
Logging	0 (0)	1 (1)	1 (1)
< 5 years old	2 (4)	0 (0)	2 (2)
Total	48 (100)	78 (100)	126 (100)

.

Table 4.3. Top 10 differentially reactive proteins recognized by all *Plasmodium vivax* malaria patients.

Gene Identification Number	**Gene Name (annotation)**
PVX_114145	merozoite surface protein 10
PVX_081830	hypothetical protein
PVX_117680	hypothetical protein, conserved
PVX_000930	sexual stage antigen s16
PVX_110935	hypothetical protein
PVX_097730	hypothetical protein
PVX_097625	merozoite surface protein 8
PVX_118705	hypothetical protein, conserved
PVX_082650	merozoite surface protein 7 (MSP7)
PVX_115450	hypothetical protein, conserved

CHAPTER 5: CONCLUSIONS

We have shown in this dissertation research that malaria transmission in the Peruvian Amazon varies between different microregions. This heterogeneous transmission pattern is a result of the human socio-demographical and behavioral characteristics that are associated with contrasting sites in this region.

Malaria transmission reflects a complex interplay among parasite, human host and vector. In the hetereogenous microgeography of the Peruvian Amazon, where occupation-related river transportation is common in addition to road travel, people move—along with their parasites—to different regions, which transports the parasites, enables new mixtures of parasites within the mosquito vector and selection for fitness and persistence in human populations. This human behavior, along with the intrinsic biology of *Plasmodium vivax* with its ability to form dormant hypnozoites that may last years until reactivation, is a major driver of malaria persistence in this region.

The research presented here shows that loggers and agriculturists are active transmitters of malaria parasites in the Peruvian Amazon. Local residents employed in these two occupations venture into the Amazon rainforest along rivers (so called riverine patterns of transmission) to work where they contract malaria at a higher rate due to the greater transmission rates in these less developed remote areas. While the exact state of malaria transmission along the rivers in the Amazon where travel-related laborers go to work is unknown, it is most likely that these areas are hyper-endemic due to

the intensity of mosquito breeding in these areas as compared to settlement or more urban areas. Malaria infections are brought back to the villages of returning residents and the malaria carriers are therefore sources of reintroduction of malaria parasites and response for the introduction of new strains in new places.

Human populations in the Peruvian Amazon villages are highly mobile, related to work and to governmental incentives to settle new land areas, both of which keeps settlements unstable. This population instability increases the risk of malaria epidemics as a continuous flow of malaria-infected laborers from high-transmission areas move to low-transmission areas. This population movement dynamic is a proximate cause of the seasonal epidemics seen in what would otherwise be low- or no-transmission areas. The secondary effects of this unique transmission dynamic can also be seen in our data. I found that subjects living with a logger or agriculturist, such as housewives and students who cohabitate with loggers and agriculturists are at a higher risk of acquiring malaria, which further strengthens our hypothesis that residents participating in these two occupations are key transmitters of malaria from regions of high transmission to regions of low transmission.

Based on the data presented here it is apparent that the Peruvian National Malaria Control Program must include residents who travel as part of their occupation and those cohabitating with them into their surveillance activities and that these groups must be considered a special high-risk

subpopulation. As such, these groups need to be provided more extensive follow-up care. In addition, as these high-risk subpopulations are one of the predominant causes of epidemics, providing these groups with prophylactic treatment should lessen the malaria burden on the community as a whole. Malaria control in these villages, therefore, could be achieved most efficiently by the deployment of targeted interventions of the identified high-risk subpopulations.

Research presented here also demonstrates the high diversity of *Plasmodium vivax* parasites in the Peruvian Amazon. Using molecular markers to identify and differentiate parasites I have shown that in the Peruvian Amazon there is a high proportion of infections caused by more than one parasite genotype. Taken together the high diversity and large number of polyclonal infections argue against the conventional wisdom that the Peruvian Amazon is a uniformly a hypo-endemic region with limited parasite diversity. The increase in predicted transmission rate using observed characteristics indicates that we lack critical knowledge of transmission intensity and entomological inoculation rates in the Peruvian Amazon. It also highlights the importance of continued work like that above that showed that areas predicted to be low-transmission due to ecological factors may in fact have higher malaria transmission rates due to socio-behavioral characteristics that have been heretofore underappreciated.

Additionally, by combining the molecular biology analysis above with

socio-demographic analysis, we were able to delineate a differential risk to relapse and to re-infection in distinct micro-geographical settings within the Peruvian Amazon. After identifying relapse and re-infections, we next sought to identify if there was a differential human immune response to these two types of infections. Our hypothesis was that a relapse infection would trigger a more directed antibody response since the immune system should have been exposed to the parasite epitopes in the initial infection. Using a protein binding microarray to assess overall antibody response after infection, I found that a relatively small set of differentially reactive *P. vivax* antigens are sufficient to identify *P. vivax* relapse and reinfections. In addition, a reinfection is associated with a higher cumulative intensity signal on the protein arrays against the complete set of reactive antigens than relapsed infection. These data indicate that a reinfection caused by a parasite not previously seen by the patient's immune system will cause a broader and more intense immune response as we had hypothesized.

The *P. vivax* antigen with the highest intensity on the protein array was PvMSP10; others top ranked antigens included MSP7, MSP8 and several hypothetical proteins, and a putatively sexual stage antigen s16. Bioinformatics analysis of the genes encoding for these antigens indicates that the differentially reactive antigens are under diversifying selection. This phenomenon is reflected by the fact that non-synonymous nucleotide changes are more common (dn/ds > 1) than expected by chance. This means that

these antigens are being evolutionarily selected to have a more diverse set of amino acids. This type of selection is most associated with immune selection in host-pathogen interactions and is an example of the “Red Queen” hypothesis which states that the parasite and host (more specifically those proteins that interact with one another) are continually evolving in response to the other. This constant co-evolution leads to diverse genes across a parasite population, which is what these data support.

The data and conclusions presented in this work have stimulated novel approaches to key questions remaining in the field about how to apply new strategies towards malaria control and elimination research. First, analysis of socio-demographics of high risk groups for malaria in the Peruvian Amazon indicate the need to carry out operational research focused on the deployment of targeted interventions to prevent malaria affecting specific groups of high-risk subpopulations, specifically agricultural and logging laborers who travel away from home and acquire malaria infection in remote places. Second, population based cohort studies in villages located in remote areas of the Peruvian Amazon indicate the need for more active malaria surveillance compared to the more convenient and conventional malaria control activities in villages surrounding the city of Iquitos where malaria is comparatively well understood. Third, entomology-focused studies are essential for understanding the mechanisms for the generation of parasite diversity, continued transmission in affected populations and the heterogeneous malaria

transmission patterns in the Peruvian Amazon region related to vector ecology; vector ecology is also highly affected by human behaviors, for example deforestation, so that malaria control and elimination strategies need to formally take into account the complexity of how vectors and human populations interact. Such considerations explain the different entomological inoculation rates (malaria transmission rates) in the Peruvian Amazon including not only the villages surrounding Iquitos but also and most importantly the places where actually villagers travel to for occupational purposes. Fourth, this work has identified new tools that will be useful to develop as new sero-epidemiological tools, based on the small set of differentially reactive *Plasmodium vivax* antigens we have found. The work presented here will directly lead, for example, to investigations to evaluate the potential of the top ranked antigens recognized by malaria patients in the Peruvian Amazon for use in sero-epidemiological determination of patterns of malaria transmission and to assess the presence—after elimination measures are initiated—of reintroduced infections. Fifth, the work presented here demonstrates the importance of routinely carrying out quantitative PCR in addition to conventional microscopy for potentially reduce malaria incidence of symptomatic/asymptomatic carriers of malaria parasites. The use of qPCR has important policy implications at the level of the Peruvian Ministry of Health in terms of what laboratory tests combined with clinical scenarios need to prompt the initiation of anti-malarial treatment. Finally, the three studies that comprise this dissertation thesis research conceptually integrate to provide compelling

and detailed evidence supporting the overarching central hypothesis of this dissertation that human behavior, mosquito ecology, and *Plasmodium vivax* biology have complex interactions that need to be addressed to lead to regional malaria elimination, and, with an eye to the future, global malaria eradication.