Malaria Molecular Epidemiology Unit

2013

The scientific activity report of the Malaria Molecular Epidemiology Unit of the Institut Pasteur du Cambodge for the period January 1st to December 31st, 2013.
RESEARCH PROGRAM
Challenges and key issues on malaria elimination: *P. falciparum* artemisinin resistance and knowledge on *P. vivax* malaria epidemiology

The Malaria Molecular Epidemiology Unit is a research structure, which enhances Pasteur Institute of Cambodia actions' in Public Health and scientific researches, alongside Cambodian national actors and regional/international partners. Founded in 2001, this unit is currently composed by 23 permanent staff: one senior scientist (Charge de recherché IP, head of the unit), 2 IPC research assistants (PhD), 3 PhD students, three research engineers, one unit administrator, one quality manager, one field/samples coordinator and twelve laboratory technicians.

1. Context

In Cambodia, malaria with an incidence of 4.07 per 1,000 population and 135 deaths in 2012 continues to be a major cause for public health and economic burden. Its control is given high priority by the government and development partners. Forest villagers in the eastern and northern provinces are at high risk of malaria, with all age groups suffering infection; children under the age of five years are at highest risk of severe disease due to their lack of immunity. Elsewhere, malaria is an occupational disease with specific high-risk groups, including forestry workers, new settlers and mobile/migrant populations who have come into forested areas, and soldiers, and their families, serving in the forests.

The five *Plasmodium species* known to cause malaria in humans (*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*) have already been described. Currently, *P. falciparum* remains the most frequent cause of malaria infections (prevalence of 63% in 2012). However, distributions of *Plasmodium species* are changing since several years, with a particularly significant rising of *P. vivax* malaria cases (from 8% in 2000 to 37% in 2012). Moreover, in areas of low transmission, a proportion of *P. vivax* infections up to 50% is commonly found. This trend, probably related to various effective strategies implemented in Cambodia against *P. falciparum* malaria, shows clearly that we had significantly underestimated the burden of other simian *Plasmodium species*.

Although there has been a steady reduction in the total number of clinically diagnosed and treated malaria cases as well as in the severe case fatality rate over the last thirteen years, morbidity and mortality due to malaria remain high compared to other countries in the region. Malaria in Cambodia is also a key contributor to anaemia, complications during pregnancy, low-birth weight and poor child growth.

In addition, multi-drug resistant strains of *Plasmodium falciparum* are common, particularly in the west of the country. As with earlier antimalarials, we are now facing to the emergence of artemisinin resistance in western Cambodia while no suitable alternative currently exists for first-line treatments of *P. falciparum* malaria. As previously observed with chloroquine resistance in the last century, artemisinin-resistant parasites represent a major threat to worldwide goals of malaria eradication and the potential to devastate sub-Saharan Africa by increasing childhood mortality.

2. Major Areas of Research since 2010

Scientific projects conducted in the unit rely on performant technical platforms including equipment for cellular culture, molecular biology and immunology. Built around the control/elimination concept in South East Asia,
projects are conducted in close collaboration with the Cambodian National Malaria Control Programme, WHO and others regional and international partners (RIIP, IPP, European and US Universities).

They are mainly focused on three major areas of research (figure 1):

1. **Supporting and evaluating the impact of strategies against malaria implemented by National Malaria Control Programmes**

2. **Conduct researches focused on *P. falciparum* artemisinin resistant parasites.**

3. **Conduct researches on vivax malaria & other emerging *Plasmodium sp.***

### 3. Major Achievements (2010-2013)

#### 3.1. Research Area 1. Supporting and evaluating the impact of the strategies implemented by NMCPs

**Investigation of undifferentiated febrile illnesses in rural Cambodia.** In the past decade, control of *falciparum* malaria has been successfully implemented in Cambodia, resulting to a significant decrease of reported malaria. The introduction and wide-use of malaria rapid diagnostic tests (RDTs) within this program has revealed a large burden of clinically suspected malaria cases in which no malaria parasites are detected. As a first step towards developing an algorithm for malaria-RDT negative fever management, a 3-year cross-sectional prospective observational study was designed to investigate the causes of acute malaria-negative febrile illness in Cambodia. A total of 1193 febrile patients and 282 non-febrile individuals were recruited from three sites in rural Cambodia. Whole venous blood, blood smear and nasopharyngeal throat swabs were collected. The samples were screened for malaria parasites by RDT, microscopy and PCR, for Leptospira, Rickettsia and O. tsutsugamushi by PCR, for Dengue- and Influenza virus by RT-PCR as well as for community acquired septicaemia by blood culture. At least one pathogen was identified in 73.2% of febrile patients. Most frequent pathogens detected by molecular diagnostics were *P. vivax* (33.4%), *P. falciparum* (26.5%), *Leptospira* (11.3%), *Influenza* viruses (7.7%), *Dengue* viruses (5.4%), *O. tsutsugamushi* (3.7%), *Rickettsia* (0.2%), and *P. knowlesi* (0.1%). A potential pathogen was identified in 873/1193 febrile patients and 114/282 non-febrile subjects. Pathogens, particularly malaria parasites and leptospirosis, were also identified in asymptomatic individuals. Clinic-based diagnosis of malaria RDT-negative cases was found to poorly predict for pathogen and appropriate treatment.

**G6PD deficiency issues: prevalence studies, diagnosis and recommendation for the safe introduction of the Primaquine in National treatment guidelines.** Controlling malaria remains a significant global health challenge,
especially in areas of low transmission which are seen as prime areas for malaria elimination. In this context, the WHO has been urging countries for many years to use primaquine for both transmission blocking of *Plasmodium falciparum*, because it kills mature gametocytes, and as anti-relapse treatment against *Plasmodium vivax* by killing liver hypnozoites. But Primaquine is not used widely because of anxiety over its well-known propensity to cause acute haemolytic anaemia in individuals with G6PD deficiency, coupled with the current logistical and fiscal impossibility of offering G6PD screening to all malaria patients. In this two-year survey, the prevalence of the G6PD deficiency and haemoglobinopathies were assessed by quantitative enzyme activity assay and haemoglobin electrophoresis, in samples collected from 2,408 confirmed malaria patients in 19 health centres throughout Cambodia. *Plasmodium falciparum* was present in 1,443 (59.9%) and *P. vivax* in 965 (40.1%) patients. Mean G6PD activity was 11.6 (CI 95%: 11.4-11.8) U/g Hb, G6PD deficiency was present in 13.9% of all patients (335/2,408) and severe G6PDd (including WHO Class I and II variants) was more common in western (158/1,732, 9.1%) versus eastern (21/414, 5.1%) Cambodia (P=0.01). Of 997/2,408 (41.4%) had a haemoglobinopathy.

In addition, we assessed a rapid diagnostic test under research and development called CareStart™ G6PD deficiency screening test (Access Bio, New Jersey, USA) by comparing its performance to quantitative G6PD enzyme activity method ('gold standard'). Blood samples (n=903) were collected from Cambodian adults living in Pailin province, western. Based on a normal haemoglobin concentration and wild-type G6PD gene, the normal values of G6PD enzymatic activity for this population ranged from 3.6 to 20.5 U/g Hg (95th percentiles from 5.5 to 17.2 U/g Hg). Ninety-seven subjects (10.7%) had <3.6 U/g Hg and were classified as G6PD deficient. Prevalence of deficiency was 15.0% (64/425) among men and 6.9% (33/478) among women. Genotype was analysed in 66 G6PD-deficient subjects and 63 of these exhibited findings consistent with Viangchang genotype. The sensitivity and specificity of the CareStart™ G6PD deficiency screening test was 0.68 and 1.0, respectively. Its detection threshold was < 2.7 U/g Hg, well within the range of moderate and severe enzyme deficiencies. Thirteen subjects (1.4%, 12 males and 1 female) with G6PD enzyme activities < 2 U/g Hg were falsely classified as "normal" by RDT. We concluded that this experimental RDT test here evaluated outside of the laboratory for the first time showed real promise, but safe application of it will require lower rates of falsely "normal" results.

Following two meeting dedicated to G6PD deficiency issues, the discussions and conclusions were reported in 2 publications. The first one from a workshop conducted in Incheon, Korea in May 2012 described the key knowledge gaps in G6PD deficiency detection and proposed certain research priorities and an action plan. The second meeting, held in Bangkok, Thailand in October 2012, was focused on challenges to the development and evaluation of G6PD diagnostic tests, and on challenges related to the operational aspects of implementing G6PD testing in support of radical cure.

An innovative tool for moving malaria PCR detection of parasite reservoir into the field. To achieve the goal of malaria elimination in low transmission areas such as in Cambodia, new, inexpensive, high-throughput diagnostic tools for identifying very low parasite densities in asymptomatic carriers are required. This will enable a switch from passive to active malaria case detection in the field. In this study, we described an innovative approach developed by our unit to detect malaria parasites carriers by PCR. DNA extraction and real-time PCR assays (real-time PCR screening and species identification) were performed in a mobile laboratory, in Rattanakiri Province, to screen approximately 5,000 individuals in less than four weeks and
treat positive cases within 24–48 hours after sample collection. An average of 240 clinical samples (and 40 quality control samples) was tested every day, six/seven days per week. 97.7% of the results were available <24 hours after the collection. The operational success of this diagnostic set-up proved that molecular testing and subsequent treatment is logistically achievable in field settings, allowing the detection of clusters of asymptomatic carriers and to provide useful epidemiological information. We concluded that the concept of the mobile laboratory could be extended to other countries for the molecular detection of malaria or other pathogens, or to culture vivax parasites, which does not support long-time delay between sample collection and culture.

3.2. Research Area 2. *P. falciparum* artemisinin resistance

**Novel phenotypic assays for the detection of artemisinin resistant *Plasmodium falciparum* malaria in Cambodia:** *in vitro* and *ex vivo* drug-response studies. In this study, we aimed to assess whether the *in vitro* ring-stage survival assay (RSA) can identify culture-adapted *P. falciparum* isolates from patients with slow-clearing or fast-clearing infections, to investigate the stage-dependent susceptibility of parasites to dihydroartemisinin in the *in vitro* RSA, and to assess whether an *ex vivo* RSA can identify artemisinin-resistant *P. falciparum* infections.

We culture-adapted parasites from patients with long and short parasite clearance half-lives from a study done in Pursat, Cambodia, in 2010 and used new *in vitro* survival assays to explore the stage-dependent susceptibility of slow-clearing and fast-clearing parasites to dihydroartemisinin. In 2012, we implemented the RSA in prospective parasite clearance studies in Pursat, Preah Vihear, and Ratanakiri, Cambodia, to measure the *ex vivo* responses of parasites from patients with malaria.

Our results showed that *in vitro* survival rates of culture-adapted parasites from 13 slow-clearing and 13 fast-clearing infections differed significantly when assays were done on 0–3 h ring-stage parasites (10.88% vs 0.23%; p=0.007). *Ex vivo* survival rates significantly correlated with *in vivo* parasite clearance half-lives (n=30, r=0.74, 95% CI 0.50–0.87; p<0.0001). We concluded that the *in vitro* RSA of 0–3 h ring-stage parasites provides a platform for biochemical and molecular characterization of artemisinin resistance and the *ex vivo* RSA can be easily implemented where surveillance for artemisinin resistance is needed.

**A molecular marker of artemisinin resistant *Plasmodium falciparum* malaria.** Following our previous findings, we were able in this study, by using whole-genome sequencing of an artemisinin-resistant parasite line from Africa and clinical parasite isolates from Cambodia, to associate mutations in the PF3D7_1343700 kelch propeller domain (‘K13-propeller’) with artemisinin resistance *in vitro* and *in vivo*. Parasites bearing mutant K13-propeller alleles were observed in Cambodian provinces where artemisinin resistance is prevalent. We also found that the increasing frequency of a dominant mutant K13-propeller allele correlates with the recent spread of resistance in western Cambodia. Strong correlations between the presence of a mutant allele (C580Y), *in vitro* parasite survival rates and *in vivo* parasite clearance rates indicated that K13-propeller mutations are important determinants of artemisinin resistance. Our conclusion was that K13-propeller polymorphism constitutes a useful molecular marker for large-scale surveillance efforts to contain artemisinin resistance in the Greater Mekong Subregion and prevent its global spread.
3.3. Research Area 3. *P. vivax* malaria challenges & other emerging *Plasmodium sp.*

**Whole genome sequencing of field isolates provides robust characterization of genetic diversity in *Plasmodium vivax.*** Here, we reported results from whole genome sequencing of five *P. vivax* isolates obtained from Malagasy and Cambodian patients, and of the monkey-adapted Belem strain. We obtained an average 70–400 X coverage of each genome, resulting in more than 93% of the Sal I reference sequence covered by 20 reads or more. Our study identified more than 80,000 SNPs distributed throughout the genome which will allow designing association studies and population surveys. Analysis of the genome-wide genetic diversity in *P. vivax* also revealed considerable allele sharing among isolates from different continents. This observation seems to be consistent with a high level of gene flow among parasite strains distributed throughout the world. Moreover, we demonstrated the feasibility to perform whole genome sequencing of *P. vivax* from field isolates and the rigorous characterization of their genetic diversity. We concluded that the catalogue of polymorphisms generated will enable large-scale genotyping studies and contribute to a better understanding of *P. vivax* traits such as drug resistance or erythrocyte invasion, partially circumventing the lack of laboratory culture that has hampered vivax research for years.

**Whole genome sequencing of field isolates reveals a common duplication of the Duffy Binding Protein gene in Malagasy *Plasmodium vivax* strains.** Until recently the Duffy-negative blood group phenotype was considered to confer resistance to vivax malaria for most African ethnicities. We and others have reported that *P. vivax* strains in African countries from Madagascar to Mauritania display capacity to cause clinical vivax malaria in Duffy-negative people. New insights must now explain Duffy-independent *P. vivax* invasion of human erythrocytes. Through recent whole genome sequencing we obtained ≥ 70X coverage of the *P. vivax* genome from five field-isolates. Combined with sequences from one additional Malagasy field isolate and from five monkey-adapted strains, we described identification of DNA sequence rearrangements in the *P. vivax* genome, including discovery of a duplication of the *P. vivax* Duffy binding protein (PvDBP) gene. A survey of Malagasy patients infected with *P. vivax* showed that the PvDBP duplication was present in numerous locations in Madagascar and found in over 50% of infected patients evaluated. Extended geographic surveys showed that the PvDBP duplication was detected frequently in vivax patients living in East Africa and in some residents of non-African *P. vivax*-endemic countries. Additionally, the PvDBP duplication was observed in travelers seeking treatment of vivax malaria upon returning home. We also observed that PvDBP duplication prevalence was highest in west-central Madagascar sites where the highest frequencies of *P. vivax*-infected, Duffy-negative people were reported. The highly conserved nature of the sequence involved in the PvDBP duplication suggested that it has occurred in a recent evolutionary time frame. Our conclusion hypothesized that PvDBP, a merozoite surface protein involved in red cell adhesion is rapidly evolving, possibly in response to constraints imposed by erythrocyte Duffy negativity in some human populations.

**De novo assembly of a field isolate genome reveals a novel *Plasmodium vivax* Erythrocyte-Binding Protein gene.** Recent sequencing of *Plasmodium vivax* field isolates and monkey-adapted strains enabled characterization of SNPs throughout the genome. These analyses relied on mapping short reads onto the *P. vivax* reference genome generated from a monkey-adapted strain. Any locus deleted in this genome would be lacking in the reference sequence and missed in previous analyses. In this study, we reported de novo assembly of a *P. vivax* field isolate genome. Out of 2,857 assembled contigs, we identify 362 contigs each containing more than 5 kb of contiguous DNA sequences absent from the reference genome sequence. These novel *P.
vivax DNA sequences accounted for 3.8 million nucleotides and contained 792 predicted genes. Most of these contigs contained members of multigene families and likely originated from telomeric regions. Interestingly, we identified two contigs containing predicted protein coding genes similar to Plasmodium red blood cell invasion proteins. One gene encoded the reticulocyte-binding protein gene orthologous to P. cynomolgi RBP2e and P. knowlesi NBPXb. The second gene harbored all the hallmarks of a Plasmodium erythrocyte-binding protein but clustered separately from all known Plasmodium Duffy-binding protein genes. Additional analyses showed that this gene was present in most P. vivax genomes and transcribed in blood-stage parasites. The result of this study complemented previous genomic analyses and took full advantage of sequence data to provide a comprehensive characterization of genetic variations in this important malaria parasite.

4. On-going projects

4.1. Research Area 1. Supporting and evaluating the impact of the strategies implemented by NMCPs

4.1.1. Major objectives

As Cambodia move towards malaria elimination, activities which aim to measure how public health programs operate over time and achieve their goals will need to shift from measuring reductions in morbidity and mortality, to detecting infections especially in asymptomatic parasite carriers and measuring transmission. Thus, the monitoring and evaluation research needs to develop tools that will replace passive surveillance of morbidity with active and prompt detection of infection, including confirmation of interruption of transmission by detecting present and past infections, particularly in mobile populations.

In this context, the projects currently conducted in the unit aim at:

- developing and implementing track and treat strategy by using high throughput real time PCR in the field (mobile laboratory unit) in hotspots areas and among populations at risk.
- (re)defining the malaria epidemiology in low transmission areas by developing molecular approaches using high volume of blood sampling.
- evaluating the structure of the parasite population to understand the parasite genes flow and monitor the spread of antimalarial drugs resistant parasites in Cambodia.
- developing tools to evaluate the malaria transmission, including serological markers and detection of gametocytes carriers.
- improving point-of-care tools to detect G6PD deficiency to facilitate the safety use of primaquine to treat falciparum malaria (gametocytes) or vivax malaria (hypnozoites).

4.1.2. Awarded on-going grants

- Title: Towards malaria elimination: effective strategies against transmission. The new challenges in South East Asia.
  Sponsor: Initiative 5% (AP-5PC-2012-02) – French Government
Period: July 2013 - July 2015

- **Title:** Repellents as added control measure to long lasting insecticidal nets to target the residual transmission in Southeast Asia: a step forwards to malaria elimination
  
  Sponsor: Bill & Melinda Gates Foundation via the Prince Leopold Institute of Tropical Medicine

  Period: October 2011 - September 2015

- **Title:** Developing the Evidence for and assessing the Malaria Elimination Efforts among Mobile Migration Workers in Plantation Settings in Cambodia and Myanmar.
  
  Sponsor: Bill & Melinda Gates Foundation via Population Services International

  Period: December 2013 - February 2015

- **Title:** Developing a screening algorithm to optimize identification of asymptomatic malaria among migrants crossing Cambodian borders (Cross Border Project)
  
  Sponsor: USAID via Malaria Consortium

  Period: July 2013 - April 2014

4.2. Research Area 2. *P. falciparum* antimalarial drug resistance and treatment

4.2.1. **Major objectives: One step beyond K13**

While we have achieved some important steps in our understanding in artemisinin resistance (ART-R), especially with the development of two major tools (*in vitro* phenotype with the RSA and molecular signatures of ART-R parasites with K13 mutations), many questions remain unanswered. They are mainly focused around three axes:

**To better understand the phenomenon of “dormancy” at cellular and molecular levels, and its relationship with the biological role of the K13 gene.**

Avenues of investigations are now opened by the discovery of the K13 gene.

- We need to identify the metabolic pathway in which it operates, especially the biological partners involved in the cell and to investigate the fitness cost of the different K13 alleles in the absence of artemisinin pressure. We have still initiated these studies with several collaborators (David Fidock, University of Columbia) to assess the impact of the most frequent K13 mutations on the biology of the parasite, its responses to oxidative stress and other antimalarials.

- We also need to develop imaging methods to quantify the number of dormant parasites in cultures treated by artemisinin derivatives. Our data have already identified that 0-3 hours ring-stages are those who best survived to artemisinins and showed that this resistance is stable. To better understand the phenomenon of dormancy, we are planning to explore various strategies for labeling parasites with fluorescent dyes to quantify by flow cytometry the percentage of artemisinin-exposed survival parasites.

- We will investigate the level of RNA expression of certain genes involved in the response to artemisinin derivatives exposure. Our work will be particularly focused on the role of the mitochondria and the apicoplast. Quantitative PCR will be developed to explore gene amplification among our samples collection.
of Cambodian isolates. This work will allow us to identify new molecular mechanisms that may be involved in dormancy and resistance to artemisinin derivatives.

- Finally, we are also planning to study the relationship between resistance to artemisinins, dormancy and oxidative stress defense mechanisms. We have already observed a cross resistance between artemisinin derivatives and other oxidant molecules like methylene blue. It seems that the same cellular mechanism associated to dormancy is involved. This work will be done in coordination with imaging observations and genomic/transcriptomic studies.

To better understand the emergence and the spread of artemisinin resistant parasites by studying the overtime selection of parasite populations maintained under drug pressure.

Our recent observations indicate that resistance to artemisinins and high survival rates in in vitro RSA assays are not only associated with a single mutation in K13 gene. Beyond the analysis of the different K13 mutant-type parasites, we have to understand how and where these mutations emerge and to investigate their ability to further spread. Is that all C580Y mutant-type parasites (the most frequent mutant allele found in Cambodia) come from the same parasite population or are they from several sub-populations which have acquired independently the same mutation? This important issue should enable us to understand the natural evolution of field parasite populations but also improve the development of effective tools used to monitor and control malaria endemcity. Finally, by analyzing neutral SNPs/microsatellite sequences around the K13 gene we will determine how many independent events have taken place to select mutants K13. This will open the door to the analysis of biological, epidemiological and environmental factors that contributed to the selection of these mutations. We will also analyze the speed of propagation of mutations in populations. These data help predict potential outbreaks and deploy measures accordingly.

To improve the surveillance of the ACT efficacy with the evaluation of the spatial distribution of ART-R parasites. The mutant type K13 alleles are markers will allow us to easily map the presence or absence of ART-R parasites in different endemic areas, alert WHO and local health authorities on the need to recommend new antimalarial regimens. Recently, it has been decided by WHO that Institut Pasteur leads a WHO reference center for the global mapping of K13 alleles. This multi-site platform, implemented in Paris, in Cambodia and in French Guiana, will be in charge to develop a central data base collecting information on K13 mutant prevalence and SOPs for detecting K13 mutations and to implement a quality assurance system in association with partners in malaria endemic countries involved in this global network. The aim will be to combine efforts to make a world map of this new molecular marker and to inform in real-time decision-makers of the best therapeutic option.

In addition, the Malaria Molecular Epidemiology Unit as head of the in vitro group of wWARN project will be in charge to organize workshops and train staff from research centers (both in Phnom Penh or locally) to facilitate the implementation of in vitro and ex vivo susceptibility testings for artemisinin derivatives and partner drug in countries aiming to control or eliminate malaria. We will provide support to implement quality assurance of in vitro susceptibility testings by providing reference strains through MR4 repository and support for external quality controls (exchange of P. falciparum parasite isolates for internal or external QC), update detailed SOPs and support for the interpretation of the results of the in vitro assays.
Lastly, in collaborations with Medicines for Malaria Venture, we will be also actively involved in projects aiming to screen new molecules. By using a panel of parasites with different in vitro susceptibility level and different genetic background, we will assess different “Late Lead Molecules” and “preclinical Molecules” in 2014.

4.2.2. Awarded active grants

- **Title: IMMERSE: Innovative Malaria M&E, Research and Surveillance towards Elimination**
  Sponsor: CDC/PMI/USAID via Malaria Consortium
  Period: November 2013 - October 2015

- **Title: Leading In vitro module of wWARN project**
  Sponsor: Bill & Melinda Gates Foundation via World Wide Antimalarial Resistance Network
  Period: January 2014 - March 2015

- **Title: Artemisinin in vitro resistance**
  Sponsor: Medicines for Malaria Venture
  Period: January 2014 - December 2014

- **Title: MaPI: Lead optimisation of original anti-malaria compounds:a synergic multi-target approach**
  Sponsor: ANR (ANR-2011-RPIB-002-01)
  Period: March 2012 - February 2016

- **Title: SOREMA: Public Interventions and Health Inequalities in Recomposed Natural and Social Ecosystems of the Mekong Sub-Region**
  Sponsor: ANR
  Period: January 2012- January 2015

4.3. Research Area 3. *P. vivax* genetic diversity, resistance and biology challenges

4.3.1. Major objectives

Malaria threatens a quarter of the 13.6 million Cambodians who live near forested areas. While *Plasmodium falciparum* is the most frequent reported cause of malaria in Cambodia, the proportion of vivax malaria has significantly increased in the last decade. The extensive use of antimalarial drugs in the region, most notably chloroquine (CQ), has led to the emergence of resistant *P. vivax* parasites which constitutes one of the greatest challenges on malaria control in Southeast Asia. Assessment of drug resistance in *P. vivax* presents unique challenges and must be considered separately from *P. falciparum*. Implementation of in vitro assays to determine drug susceptibility is complicated by the difficulty to routinely culture *P. vivax*. *In vivo* assessments of drug resistance in *P. vivax* are also problematic since recurrence of malaria after treatment can be caused by i) parasite multiplication after incomplete elimination, ii) re-infection by new parasites and iii) release of dormant parasites from the liver, a mechanism not observed in *P. falciparum*. These difficulties, combined with
limited information on the genetic diversity of *P. vivax*, greatly limit our understanding of drug resistance in this parasite.

We have recently sequenced the entire *P. vivax* genomes from field isolates from Cambodia. Using recent development in sequencing technologies, we obtained 70-400 X coverage of the genomes and >93% of the nucleotides covered by more than 20 reads. Our data provided a first characterization of the genome-wide genetic diversity in *P. vivax*, at SNPs and sequence rearrangements. In addition, we identified the presence of multiple *P. vivax* strains in each infected blood sample and were able to differentiate them by reconstructing bioinformatically individual haplotypes at highly polymorphic loci. We propose to build on this experience to characterize the genetic diversity of *P. vivax* in Cambodia and identify DNA polymorphisms underlying drug susceptibility and resistance. In collaboration with David Serre (Cleveland Clinic), we will combine modern fieldwork with population genomic analyses to address the following outstanding issues:

**To characterize the genetic diversity of *P. vivax* in Cambodia.** Understanding the structure and dynamics of the parasite population is essential for monitoring the emergence and spread of drug resistance. We propose to study isolates from symptomatic and asymptomatic *P. vivax*-infected individuals recruited in sites across Cambodia. First, we will characterize the number and diversity of *P. vivax* strains present in each individual by very deep re-sequencing (>20,000 X) of five highly polymorphic loci, including the *Duffy binding protein* and the *circumsporozoite surface protein* genes. These data will allow us to reliably differentiate *P. vivax* strains and to test i) whether the parasite population is geographically stratified, ii) whether vivax infections show seasonal patterns, and iii) whether the number of strains in symptomatic and asymptomatic individuals differs. Second, we will sequence the entire genome from field isolates at high coverage (>50 X) to characterize the genome-wide patterns of genetic diversity. These data will allow us to perform state-of-the-art population genomic analyses to confirm our stratification analyses and determine the amount of gene flow among Cambodian *P. vivax*. We will also scan the *P. vivax* genome for recent positive selection events that may identify drug resistant loci.

**To identify genetic polymorphisms associated with drug resistance.** Despite alarming reports of CQ resistant *P. vivax* parasites in Cambodia and other Southeast Asian countries, we know very little about the genetic bases of this resistance. We will follow infected solely with *P. vivax* for 42 days after CQ treatment and examine the evolution of the parasitemia. First, we will monitor changes in the relative proportion of the strains in each patient using our deep re-sequencing assay. Combined with microscopic determination of the parasitemia, this analysis will provide quantitative estimates of the CQ susceptibility of each *P. vivax* strain. Second, we will collect additional blood samples from patients who showed evidence of treatment failure, and use our deep re-sequencing assay to differentiate recrudescence from new infections. We will also measure the drug concentration in blood to confirm drug resistance, and perform short-term cultures of *P. vivax* directly in the field, using a mobile laboratory, to assess *ex vivo* the susceptibility of the parasites to CQ. We will then link these different estimates of drug resistance with genome-wide diversity data to identify DNA polymorphisms statistically associated with drug resistance.

In addition, a second project on *Plasmodium vivax* will be conducted in collaboration with Peter Zimmerman (Case Western University, Cleveland, Ohio) and Arsene Ratsimbasoa (National Malaria Control Programme in
Madagascar). The study will aim to understand how *Plasmodium vivax* has gained capacity to infect erythrocytes from Duffy-negative people based on our recent findings, to leading to the following hypotheses:

(1) In Madagascar Duffy (-) and Duffy (+) individuals are equally susceptible to *P. vivax* infection, but differentially susceptible to different *P. vivax* strains, or (2) *P. vivax* has evolved alternative erythrocyte invasion pathways to enable infection of Duffy (-) erythrocytes, or (3) *P. vivax* strains exhibit differences in erythrocyte binding and invasion efficiency.

Our hypotheses will be tested through the following specific aims.

**Identify associations between *P. vivax* strains and susceptibility of Duffy (-) people to *P. vivax* blood-stage infection and clinical malaria.** We will determine the relative susceptibility of Duffy (-) and Duffy (+) people to *P. vivax* infection and disease in communities where our preliminary study found the highest prevalence of *P. vivax* infections in Duffy (-) individuals.

**Assess interactions between *P. vivax* erythrocyte binding ligands and human erythrocytes that influence merozoite attachment and invasion of host red cells.** Studies will first focus on the PvDBP allele(s) (single-copy and duplicated) observed to be present in *P. vivax*-infected Duffy (-) and Duffy (+) people (Malagasy and Cambodian). Following cloning, over-expression and purification of recombinant variant PvDBP alleles (Malagasy and Cambodian), we will evaluate erythrocyte antigen binding of variant PvDBP alleles across a range of concentrations. Through previously developed assays we will test erythrocyte binding of PvDBP variants to Duffy (-) and Duffy (+) red blood cells that have been screened for standard blood group specificities. Alternative invasion ligands of interest will include the *P. vivax* reticulocyte binding proteins (PvRBP), apical membrane antigen-1 (PvAMA-1) and other micronemes and rhoptery proteins.

**Classify in vitro invasion pathways of Malagasy *P. vivax* patient isolates for Duffy (-) and Duffy (+) erythrocytes.** *In vitro* *P. vivax* invasion studies will follow methods used with success by Grimberg et al. *P. vivax* strains will first be classified as *Independent or Dependent* on PvDBP following exposure to PvDBP antibodies (mono- and poly-clonal). Further classification *P. vivax* invasion pathways will include exposure of parasite isolates to Duffy antigen variants (e.g. Fya vs Fyb), red cell enzyme treatments, and additional *P. vivax* antigen-specific antibodies.

### 4.3.2. Awarded active grants

- **Title: Madagascar *P. vivax* Invasion of Duffy-Negative Red Cells**  
  Sponsor: NIH/NIAID (R01 AI097366)  
  Period: November 2013 - October 2018

- **Title: Genomic Analyses of *Plasmodium vivax* Responses to Antimalarial Drugs in Cambodia**  
  Sponsor: NIH/NIAID (R01 AI103228)  
  Period: May 2013 - April 2018
COLLABORATIONS & SCIENTIFIC PARTNERSHIPS

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Institut Pasteur de Guyane Française E. LEGRAND, L. MUSSET
**PUBLICATIONS LIST**

### 2013 – 19 publications

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2012 – 10 publications


2011 – 5 publications


