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Contribution of molecular tools to malaria elimination in Vietnam

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Abbreviations

ACD	Active Case Detection
ACPR	Adequate Clinical and Parasitological Response
ACT	Artemisinin-based Combination Therapy
AOR	Adjusted Odds Ratio
AQ	Amodiaquine
ARI	Annual incidence rate
ART	Artemisinin
AS	Artesunate
bp	Base pair
BSA	Bovine Serum Albumin
bw	Body weight
CHC	Commune Health Central
CI	Confidence Interval
CQ	Chloroquine
CQR	Resistance to chloroquine
D	Day
DDT	Dichlorodiphenyltrichloroethane
DHA	Dihydroartemisinin (DHA)
DHA-PPQ	Dihydroartemisinin-piperaquine
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
DOT	Directly Observed
ELISA	Enzyme-Linked ImmunoSorbent Assay
ETF	Early Treatment Failure
FIND	Foundation for Innovative New Diagnostics
FOI	Force of Infection
FPBS	Filter Paper Blood Samples
F_{ST}	Genetic differentiation
G6PD	Glucose-6-Phosphate Dehydrogenase
GF	Global Fund
GIS	Geographic Information System
GMEP	Global Malaria Eradication Programme
GMS	Greater Mekong Sub-region
<i>He</i>	Expected Heterozygosity
HIV	Human Immunodeficiency Virus
HRP2	Histidine Rich Protein II
IC ₅₀	50% Inhibitory Concentration
ICEMR	International Center of Excellence of Malaria Research
IMPEs	Institute of Malariology, Parasitology and Entomology Ho Chi Minh and Quy Nhon cities
IQR	Interquartile Range

IRS	Indoor Residual Spraying
I_A^S	Standardized Index of Association
ITN	Insecticide treated nest
K13	Kelch propeller domain
LAMP	Loop-mediated isothermal Amplification
LD	Linkage Disequilibrium
LF	Lumefantrine
LIHNS	Long Lasting Insecticide Hammock Nets
LLIN	Long Lasting Insecticidal Net
LM	Light Microscopy
LTF	Late Treatment Failure
MgCl ₂	Magnesium Chloride
MoH	Ministry of Health
MOI	Multiplicity of Infection
MQ	Mefloquine
MSAT	Mass Screening and Treatment
NASBA	Nucleic Acid Sequence Based Amplification techniques
NIMPE	National Institute of Malariaology, Parasitology and Entomology
NMCP	National Malaria Control Programme
p	P value
<i>P. falciparum</i>	<i>Plasmodium falciparum</i>
<i>P. knowlesi</i>	<i>Plasmodium knowlesi</i>
<i>P. malariae</i>	<i>Plasmodium malariae</i>
<i>P. ovale</i>	<i>Plasmodium ovale</i>
<i>P. vivax</i>	<i>Plasmodium vivax</i>
PCD	Passive Case Detection
PCR	Polymerase Chain Reaction
PCR–RFLP	PCR–Restriction Fragment Length Polymorphism
PCT	Parasite clearance time
pLDH	Parasite Lactate Dehydrogenase
POC	Point of Care
PPQ	Piperaquine
PQ	Primaquine
PvCQR	Resistance to chloroquine in <i>P. vivax</i>
qPCR	Quantitative Polymerase Chain Reaction
RBM	Roll Back Malaria Partnership
RDT	Rapid Diagnostic Test
rfu	Relative fluorescence unit
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
SEA	Southeast Asia
SnM- PCR	Semi-nested multiplex Malaria PCR

SNPs	Single nucleotide polymorphisms
SP	Sulfadoxin–pyrimethamine
SSU rRNA	Small Subunit ribosomal Ribonucleic Acid
TAE	Tris-Acetate-EDTA
TB	Tuberculosis
TES	Therapeutic Efficacy Studies
VHW	Village Health Workers
WBC	White Blood Cells
WGS	Whole genome sequencing
WHO	World Health Organization

Table of contents

Abbreviations	1
Table of contents.....	5
Summary	7
Samenvatting	11
Chapter 1: Introduction.....	15
Chapter 2: Literature review on malaria	37
Chapter 3: Short report: A modified semi-nested multiplex malaria PCR (SnM – PCR) for the identification of the five human <i>Plasmodium</i> species occurring in Southeast Asia	129
Chapter 4: Human <i>Plasmodium knowlesi</i> infections in young children in central Vietnam	143
Chapter 5: Marked age-dependent prevalence of symptomatic and patent infection and complexity of distribution of human <i>Plasmodium</i> species in central Vietnam .	161
Chapter 6: Population genetics of <i>Plasmodium vivax</i> in four rural communities in Central Vietnam	189
Chapter 7: Delayed parasite clearance after treatment with dihydroartemisinin – piperaquine in <i>Plasmodium falciparum</i> malaria patients in central Vietnam	225

Chapter 8: Severe malaria not responding to artemisinin derivatives in man returning from Angola to Vietnam	257
In response: Severe malaria not responding to artemisinin derivatives in man returning from Angola to Vietnam	269
Chapter 9: General discussion	275
Curriculum vitae	319
Publications	320
Acknowledgements	323

Summary

The remarkable increase of financing and coverage of malaria control programmes has resulted in a large reduction of malaria incidence and mortality in Vietnam during the last decade. As a consequence, the proportions of asymptomatic carriers and carriers with parasitemias below the detection of microscopy have increased dramatically. Moreover, the spread of artemisinin resistant strains in the Great Mekong Sub-region is a serious threat to the current achievements of malaria control with probable global consequences. Therefore, more sensitive diagnostics and new strategies for prevention, diagnosis and treatment are required to maintain recent achievements and reach the malaria elimination goal.

This thesis focuses on the most prevalent malaria parasites in Vietnam, *Plasmodium falciparum* and *Plasmodium vivax*. It aims to understand the contribution of molecular tools for enhancing detection of malaria infection, understanding malaria transmission and improving surveillance of antimalarial drug resistance in Vietnam. These aims contribute to the country's ultimate aim of eliminating malaria by 2030.

Following detection of *Plasmodium knowlesi* parasites in Vietnam in 2009, a modified Semi-nested multiplex Malaria PCR (SnM-PCR)-based method was developed and validated to additionally identify *P.knowlesi*. The technique maintained the original sensitivity and specificity of SnM-PCR, but allowed identification of all five human

malaria parasites in three PCR steps. It is a sensitive method that is suitable for low-income countries, where budgets are limited.

The human malaria reservoir in a forested area of central Vietnam was determined by analysing 2,303 blood samples using the SnM-PCR technique. The positive rate was two-fold higher by PCR than light microscopy, with a remarkable proportion of mixed infections (25% of all infections), particularly of *P.malariae* and *P.ovale*. Such infections had previously rarely been reported. Moreover, 81.5% of malaria infections were asymptomatic, and asymptomatic carriage occurred in all age groups. Therefore, it was concluded that the malaria transmission in Vietnam is underestimated and involves a more complex species distribution than expected. Thus, diagnostic and therapeutic policies may have to be adapted accordingly.

The genetic complexity of *P.vivax* in four rural communities in central Vietnam was evaluated by analyzing 14 polymorphic loci. All samples were first analyzed by species-specific PCR. Samples confirmed as mono *P.vivax* infections were genotyped. The results showed moderate genetic diversity and a high rate of polyclonal infection (71.3%). The presence of linkage disequilibrium (LD) was detected in whole populations and in each community, suggesting gene flow within and among communities. 101 haplotypes were found, of which 84 were defined as unique. The sharing of haplotypes was quite common in these four communities, with low genetic differentiation being observed.

Regarding drug resistance, the focus has been on assessing resistance towards the most effective and commonly used antimalarial drug class: Artemisinin-based Combination

Therapies (ACTs). We documented the efficacy of the recommended first-line malaria treatment (Dihydroartemisinin–piperaquine; DHA-PPQ) in Vietnam. A total of 89 *P.falciparum* infected uncomplicated patients were monitored in a 42-day *in vivo* and *in vitro* efficacy study in Quang Nam province. The findings indicated that despite a high Adequate Clinical and Parasitological Response (ACPR: 97.7%), the day three (D3) positivity reached 29.2%. This led to Quang Nam being considered as a Tier I province (a province with evidence of ART resistance) by the World Health Organization (WHO) in 2013. The study is also one of the first in Vietnam to assess kelch propeller domain (K13 propeller)-region mutants for tracking of artemisinin resistance. The I534T mutation was the most frequent (80.7%), followed by Y493H (1.3%) and the C580Y and R539T mutations were not detected. Interestingly, 47.8% of I534T mutations on D0 were associated with delayed parasite clearance ($PCT \geq 72h$).

Next, we described a case report of malaria in a man who had returned to Vietnam after three years in Angola, who did not respond to intravenous artesunate and clindamycin, nor to an oral artemisinin-based combination. The patient was confirmed as being mono infected with *P. falciparum* by species-specific PCR, and genotyping results showed that the patient had a polyclonal *P. falciparum* infection with ≥ 2 clones persisting from admission through day 10 of hospitalization. The case report provided evidence of early treatment failure of *P. falciparum* with ACTs. This finding increases the concerns in renewing the effort to predict, detect, and mitigate the threat of antimalarial drug resistance in Africa.

In conclusion, in areas of low transmission such as Vietnam, implementation of molecular methods in control and elimination programs increases the efficiency of surveillance strategies. This should contribute to the containment and elimination of antimalarial-resistant parasites across the Mekong Region. It will also increase efficiency in detecting malaria infections (particularly subpatent infections); both essential components to ensure progress towards malaria elimination.

Samenvatting

De significante stijging van de fondsen en activiteiten voor malaria controle heeft geleid tot een grote daling van malaria incidentie en sterfte in Vietnam in het afgelopen decennium. Dientengevolge zijn de verhoudingen van zowel sub-microscopische en asymptomatische infecties enorm toegenomen. Bovendien is de verspreiding van artemisinine resistentie in de “Great Mekong” Sub-regio een ernstige bedreiging voor de bereikte resultaten in malaria bestrijding. Daarom zijn er meer gevoelige diagnostica en nieuwe strategieën nodig voor de preventie, diagnose en behandeling van malaria in deze gebieden. Dit om de recente resultaten te bestendigen en het ultieme doel van malaria eliminatie te bereiken.

Dit proefschrift focust op de meest voorkomende malariaparasieten in Vietnam, *Plasmodium falciparum* en *Plasmodium vivax*, en wil, in Vietnam, de potentiële bijdrage van moleculaire diagnostica situeren om de detectie van malaria-infectie te verhogen, de transmissie beter te begrijpen, en het monitoren van antimalaria geneesmiddelen resistentie te verbeteren. Deze objectieven zijn in lijn met het ultieme doel van malaria eliminatie van malaria in Vietnam tegen 2030

Na de ontdekking van de aanwezigheid van *Plasmodium knowlesi* parasieten in Vietnam in 2009, werd de semi-nested PCR multiplex Malaria (SnM-PCR) gebaseerde methode gemodificeerd en verder ontwikkeld en gevalideerd om ook *P.knowlesi* te identificeren. De techniek handhaafde de oorspronkelijke gevoeligheid en specificiteit van SnM-PCR,

maar identificeert nu de 5 menselijke malariaparasieten in een drie-stappen-PCR. Het is een gevoelige methode die ook geschikt is voor met lage-inkomens-landen, waar budgetten beperkt zijn.

In een bosrijke omgeving van centraal Vietnam werd het menselijke malaria reservoir bepaald door de analyse van 2,303 bloedmonsters met de SnM-PCR-techniek. Het resultaat was twee maal meer positief door middel van PCR dan microscopie, met een opmerkelijke hoeveelheid gemengde infecties (25% van alle infecties), met name *P.malariae* en *P.ovale*. Dergelijke infecties waren zelden eerder gemeld. Bovendien waren 81,5% van de malaria-infecties asymptomatisch en deze kwamen voor in alle leeftijdsgroepen. Dus hierbij toonden we aan dat de malariaendemiciteit in Vietnam onderschat wordt, en veel complexer is dan eerder werd gedacht. Daarom moeten het diagnostische en therapeutische beleid daarop worden aangepast.

De genetische complexiteit van *P.vivax* werd geëvalueerd in 4 landelijke gemeenschappen in het centraal Vietnam door het analyseren van 14 polymorfische loci. Alle stalen werden eerst geanalyseerd met species specifieke PCR, en de bevestigde mono-infecties met *P.vivax* infecties werden dan gegenotypeerd. De resultaten vertoonden tekenen van matige genetische diversiteit en een hoge proportie van polyklonale infecties (71,3%). De aanwezigheid van "linkage disequilibrium" (LD) werd gedetecteerd in de bevolking wat genetische uitwisseling binnen en tussen de verschillende bevolkingsgroepen suggereert. Honderd en één haplotypes werden gevonden, waarvan er 84 werden gedefinieerd als uniek. Het delen van haplotypes was

heel gebruikelijk in deze vier bevolkingsgroepen, waar een lage genetische differentiatie werd waargenomen

Met betrekking tot parasiet resistentie, focusten we op de meest effectieve en meest gebruikte antimalaria geneesmiddelen: artemisinine gebaseerde combinatietherapieën (ACTs). Het hoofddoel was om de werkzaamheid van de eerstelijns malariabehandeling (Dihydroartemisinine-piperaquine) (DHA-PPQ) te documenteren in Vietnam. Een totaal van 89 *P. falciparum* geïnfecteerde patiënten zonder complicaties werd voor 42 dagen in een *in vivo* en *in vitro* effectiviteitsstudie in de provincie Quang Nam opgevolgd. De bevindingen gaven aan dat ondanks een “adequate klinische en Parasitological Response” (ACPR) (97,72%), de dag drie (D3) positiviteit toch nog 29,2% was. Daardoor werd Quang Nam in 2013 door de World Health Organization (WHO) beschouwd als een zogenaamde Tier I provincie (een plaats met tekenen van resistentie tegen ACTs). De studie is ook een van de eerste in Vietnam om Kelch propeller (K13 propeller) domein mutanten te beoordelen voor het opvolgen van artemisinine resistentie. De I534T mutatie was de meest voorkomende (80,7%), gevolgd door Y493H (1,3%), met geen gedetecteerde C580Y en R539T mutaties. Interessant is dat 47,8% van de I534T mutaties bij de start van de geassocieerd werden met de vertraagde parasietverwijdering (PCT \geq 72h)

Vervolgens beschreven we een klinisch malaria geval van een man die na 3 jaar verblijf in Angola naar Vietnam was teruggekeerd. Deze man reageerde niet op een intraveneuze artesunaat en clindamycine behandeling, noch op een mondelinge

artemisinine combinatie behandeling (ACT). Species-specifieke PCR bevestigde een mono-geïnficeerd met *P.falciparum* en genotypering toonde aan dat de patiënt een polyklonale *P.falciparum* infectie had met ≥ 2 klonen die persistent waren van ziekenhuisopname tot 10 dagen na deze opname. Dit klinisch rapport bewijst een vroeg falen van de *P.falciparum* ACT behandeling en voedt de bezorgdheid opnieuw een inspanning te leveren om de dreiging van antimalaria resistentie in Afrika te voorspellen, op te sporen, en in te perken.

Samenvattend, in gebieden met een lage transmissie, zoals Vietnam, verhoogt de invoering van moleculaire methoden de efficiëntie van surveillance strategieën. Zeker voor de controle en de eliminatie programma's die gericht zijn om de spreiding van anti-malaria resistente parasieten over de Mekong regio te beperken. En verder is de verhoging van de efficiëntie van malariaparasiet detectie (in bijzonder deze met een lage parasiet bloed waarden) van essentieel belang om verder te evolueren naaraan daadwerkelijke malaria eliminatie.

Chapter 1

Introduction

1. Malaria in Vietnam: history and current situation

Vietnam has a long history of malaria control and elimination. In 1958, the first national malaria eradication program began in northern Vietnam. It included vector control based on indoor residual spraying (IRS) using dichloro-diphenyl-trichloroethane (DDT) and case management with quinine. Despite the 20-year war between 1955 and 1975, a remarkable reduction in malaria morbidity and mortality was observed during this period. After its reunification in 1976, the programme was expanded to the whole country [1,2]. Unfortunately, at the end of the war, the Vietnamese health system across the country was disrupted by massive shortages of both malaria treatments and insecticides. This led to an upsurge of malaria cases. By 1991, malaria incidence reached a peak of more than 1 million clinical cases and 4000 deaths annually [3]. This led to a paradigm shift from elimination to control strategies following WHO recommendations [2]. During the 1990's, the wide-scale use of newly adopted control interventions such as treatment with artemisinin-based therapy, distribution of insecticide treated bed nets (ITNs) and indoor residual spraying (IRS) contributed to a reversal of the trend [2]. Between 1991 and 1997, malaria mortality was reduced by 97% (from 4,646 to 152 deaths annually), malaria morbidity by 59% (from over 1 million to 445,200 clinical cases annually) and the number of outbreaks by 92% (from 110 to 10) [2,3]. This downward trend has since continued with 19,252 malaria cases and 3

malaria deaths reported in 2015 [4]. Currently, malaria transmission mainly occurs in remote and forested areas of central and central-southern Vietnam, populated by ethnic minorities with forest-related occupations (slash-and-burn agriculture, rubber plantations, logging and mining, etc.). These areas also face important seasonal population movements for economic purposes, which facilitates the spread of malaria parasites towards different regions and across international borders to countries, some of which have eliminated transmission [5-9].

In Central Vietnam, the main malaria vector is *Anopheles dirus sensu stricto*, a sylvatic and highly anthropophilic species, with early evening and outdoor biting and resting behavior. Consequently, standard vector control measures such as ITNs and IRS have been reported to be less effective [6,10-12]. This decreased protection is further aggravated by the low perception of the malaria risk among local populations [11].

P.falciparum has traditionally been the predominant human malaria species in Vietnam, accounting for more than 70% of all malaria infections between 2006 to 2010 [13]. However, in the past five years, control measures have had a stronger effect on *P.falciparum* than *P.vivax*, resulting in a shift in species prevalence with more than half (51.0%) of all infections due to *P.vivax* in 2015, while 46.0% were caused by *P.falciparum* and the remainder by *P.malariae*, *P.ovale* and mixed infections [4]. *P.knowlesi*, the so-called “fifth human malaria species” [14], has recently been identified in humans as well as in *An. dirus* specimens in different central Vietnam provinces [15,16], suggesting anthrozoootic behaviour between human and simian hosts in forested areas.

2. Malaria elimination goals and challenges in Vietnam

According to the WHO, malaria elimination is defined as the interruption of local mosquito-borne malaria transmission *i.e.* the reduction to zero of the incidence of malaria infection in a defined geographical area. Unlike malaria control, *i.e.* the reduction of the burden of disease to a level where it is no longer a public health problem, elimination requires the active detection and effective treatment of all malaria infections and continued measures to prevent re-introduction and re-establishment of transmission [17].

By the end of 2011, twenty years after switching from eradication to control, Vietnam made another strategic move in its fight against malaria, and officially endorsed malaria elimination, aiming for a malaria-free country by 2030 [18-20]. The current strategic plan (2011-20) aims at controlling malaria in areas of high (>5 confirmed cases/1,000/year) and moderate (1-5 annual cases/1,000/year) transmission, while eliminating it from areas of low transmission (<1 annual case/1,000/year). The latter currently covering 40 provinces [18]. Following the new malaria stratification map issued in 2014 by the NMCP (Figure 1), 1.3% of the country's population (*i.e.* 1,167,628 individuals in 240 communes) was still living in high endemic areas, 4.7% in moderate transmission areas, and 8.2% in low transmission areas. More than half of all communes (52.4%=5,840/11,152) were reported as malaria free (=absence of main malaria vectors and no locally transmitted malaria case) and 30% had no indigenous cases reported (no local transmission) in the past five years [5].

Control and elimination strategies are implemented depending on the local transmission characteristics. In all areas with malaria transmission, malaria diagnosis should be confirmed either by rapid diagnostic tests (RDT) or light microscopy (LM) before treatment. The recommended first-line treatment is an artemisinin combination therapy (ACT), *i.e.* dihydroartemisinin-piperaquine (DHA-PPQ), and impregnated bed-nets as well as health education campaigns are deployed. In areas of low transmission, active case detection (ACD) and prompt treatment is encouraged, whereas directly observed treatment (DOT) is required in areas where artemisinin resistance has been reported [21]. Besides the annual bed-net impregnation campaigns, long-lasting insecticidal nets (LLINs) as well as hammock nets (LLIHNs) are distributed free of charge to high-risk groups (forest workers). IRS is performed in principle once a year in moderate and high transmission areas, and twice a year, together with bed-net impregnation, in remote areas where more than 80% of indigenous populations do not use ITNs [5].

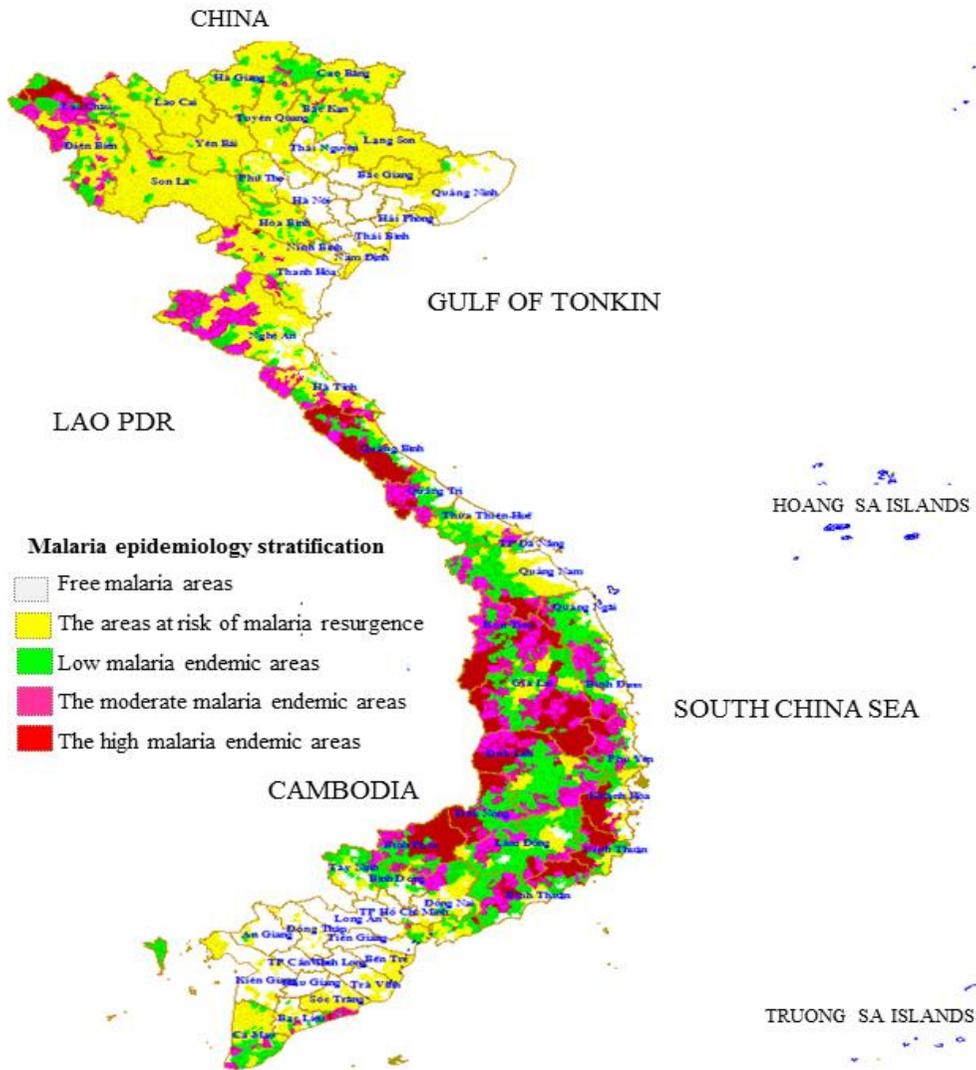


Figure 1. Malaria epidemiology stratification in Vietnam 2014 [5].

“Free malaria areas”=no malaria transmission in this area; “The areas at risk of malaria resurgence”= former malaria endemic areas where no local transmission was reported in the past 5 years; “Low malaria endemic areas”= annual malaria incidence in last five years (2009 - 2013) < 1/1.000 pop.year; “Moderate malaria endemic areas”: annual malaria incidence between 1-5/1.000 pop.year; “High malaria endemic areas”= annual malaria incidence \geq 5 /1.000.pop.year [5] .

Despite the encouraging overall reduction in malaria morbidity and mortality, the NMCP is still facing several challenges to eliminate malaria, such as forest malaria, the “hidden” nature of the human parasite reservoir, the emergence of anti-malaria drug resistance, the changes in mosquito vector behavior and their resistance to insecticides, and uncontrolled population movements [7,18,22-26].

Indeed, in Central Vietnam the standard vector control measures mentioned above are constantly challenged by the difficulty of applying these measures in rudimentary forest plot huts, where the local populations (often minority groups) spend most of their time working and sleeping [12]. In addition, mosquito resistance to pyrethroids has emerged in recent years with increased resistance to alphacypermethrin and lambda-cyhalothrin observed in malaria vectors such as *An. epiroticus*, *An. sisenensis*, and *An. vagus* [4,8,27], while other species such *An. minimus*, *An. philippinensis*, *An. subpicus* or *An. tessellatus* have shown increased tolerance levels to these compounds [8]. In addition, the behavioral adaptations reported in both *An. dirus* and *An. minimus* towards an earlier and both outdoor and indoor feeding preference, is of great concern as this is likely to further impact negatively on the effectiveness of vector control tools [28].

Recently, a high occurrence of asymptomatic and sub-microscopic infections has been reported in remote forested areas of central Vietnam when using highly sensitive diagnosis tools such as the polymerase chain reaction (PCR) [7,29]. Compared to LM, the use of molecular tools has exposed a much larger and diverse parasite population

with a higher proportion of *P. vivax* and mixed infections (some of them including *P. knowlesi*) [15, 16].

In Vietnam, delayed parasite clearance following treatment with DHA-PPQ was first reported in 2004 in the south-western Binh Phuoc province [30] and by 2016, resistance had already spread to six provinces, *i.e.* Quang Nam, Binh Phuoc, Dak Nong, Khanh Hoa, Ninh Thuan and Gia Lai, classified by the WHO as “Tier I area” (= “credible evidence of artemisinin resistance with day-3 positivity rate >10%”) [31, 32]. Even though therapeutic efficacy studies (TES) in Vietnam still show cure rates generally above 90% [22, 33], recent reports of increased treatment failure in Cambodia [34] and the confirmation of *P. vivax* resistance to chloroquine in Vietnam [24] constitute an alarming scenario for malaria elimination goals.

Finally, human mobility significantly impacts malaria transmission in Vietnam, as 19.2% of all reported malaria cases in 2015 were identified in travellers, including 7.9% among internal migrants (between different provinces of the country) and 11.3% from international migrants, mostly from Africa and neighboring countries such as Laos and Cambodia [4]. In malaria free- and areas at risk of resurgence, imported malaria cases represent a threat for malaria outbreaks and the re-establishment of local malaria transmission.

3. The use of molecular tools to support control and elimination strategies in Vietnam

In Vietnam, light microscopy (LM) remains the gold standard for malaria diagnosis in routine health care practice, and is the diagnostic tool typically used in most commune health centers (CHC) located in malaria endemic areas (3000 microscopic points/1864 endemic communes). Microscopists' performances and skills are evaluated annually both at the provincial (by Provincial Malaria Centers) and national levels. At the provincial level, the National Institute of Malariology, Parasitology and Entomology (NIMPE) in Hanoi evaluates the performance of 28 Provincial Malaria Centers in North Vietnam, while central and southern provinces are directly supervised by regional Institutes of Malariology, Parasitology and Entomology (IMPEs), located respectively in Quy Nhon and Ho Chi Minh cities. Expert technicians' skills at NIMPE and IMPEs are regularly re-evaluated by the WHO [5,8,35]. Nevertheless, maintaining an optimal quality of LM diagnosis at all levels of the health care system represents a challenge both financially and in terms of human resources [36]. Numerous and regular operational problems such as staff and reagents shortage, and poor equipment maintenance, among others, impair the quality and coverage of LM diagnosis, especially in remote areas. Therefore, in the past ten years, and with the support of the Global Fund programme, rapid diagnosis tests (RDTs) have become increasingly available in those provinces with higher transmission of malaria [37,38] and have substantially contributed to further scaling up early diagnosis and effective treatment.

The main advantages of RDTs over LM include the following: (i) RDTs are easy to use and can be performed by trained village health workers (VHWs) who are allowed to administer antimalarial treatment after parasitological confirmation with RDTs [39,40]; (ii) RDTs can be read in approximately 5-15 minutes, compared to the 45 minutes needed to read one slide by a well-trained microscopist, (iii) RDT sensitivity and specificity is comparable to that of routine LM diagnosis [41], and (iv) the use of RDTs is independent of electricity or sophisticated equipment. However, due to a large number of asymptomatic and sub-microscopic malaria infections in the region, LM and RDTs are not sufficiently sensitive to detect the human reservoir of infection. Therefore, to achieve the national elimination milestone by 2030, the NMCP requires more sensitive tools able to detect low-density infections and accurately monitor transmission.

Molecular tools are able to identify low density (*i.e.* <1 parasite/ μ l) and mixed species infections [42,43], which are often missed or misdiagnosed by LM and RDTs, and assess the effectiveness of interventions and monitor drug resistance [44,45]. In Vietnam, despite the high proportion of asymptomatic/sub-microscopic infections, PCR-based techniques are currently used only in research settings and at research centers such as NIMPE or IMPEs. Thus, the development of sensitive, high-throughput and low-cost molecular tools that can be deployed in remote areas with minimum infrastructure, is urgently needed in order to achieve malaria elimination in Vietnam and worldwide.

With malaria elimination back on the global agenda, it is essential to better understand the transmission dynamics of malaria parasites, and analyze their population structure

and genetic diversity, in order to design meaningful and effective strategies and accurately measure their impact. To this end, high-throughput genotyping tools targeting microsatellites, single nucleotide polymorphisms (SNPs), and polymorphic genes, have been widely used during the last decade to characterize parasite populations both at the local and global levels [46-48].

Therefore, mapping population structure and the parasite transmission prior to the roll-out of interventions is crucial as a benchmark against which control and elimination targets can be measured [49]. In addition, genotyping MS markers is a useful surveillance tool to track routes of transmission and gene flow, and identify imported malaria cases that may result from population movements across or within borders [50]. As a clinical application, genotyping tools are used to monitor drug resistance, since parasite recurrences can be classified as reinfections or recrudescence [51, 52]. Combinations of MS and resistance markers have been used to investigate the emergence and spread of drug-resistant parasites [53]. Finally, population structure of genes under immune selection provides important information for vaccine development [50, 54, 55].

Owing to the growing importance of *P. vivax* infections in Vietnam, efforts to eliminate malaria and monitoring and surveillance tools to prevent reintroduction must include this parasite species.

In order to understand the contribution of molecular tools to the current malaria elimination efforts in Vietnam, our research aimed at assessing their added-value in

characterizing the human parasite reservoir, understanding transmission dynamics of malaria infections, and monitoring anti-malaria drug resistance.

4. Objectives and organisation of the thesis

4.1. Objectives

The overall objective of the thesis is to generate evidence on the contribution of molecular tools to malaria elimination efforts in Vietnam.

Three specific objectives were defined as follows:

1. To develop and apply PCR assays to characterize the human malaria reservoir in forested areas of Central Vietnam and identify *P. knowlesi* infections;
2. To characterize the baseline genetic diversity and population structure of *Plasmodium vivax* parasite populations in four rural communities of central Vietnam;
3. To investigate the development and spread of *P. falciparum* parasites resistance to artemisinin combination therapies in central Vietnam and in a case of a migrant worker with severe malaria and not responsive to artemisinin derivatives;

4.2. Organization of the thesis

This thesis is organized into eight chapters as follows:

Chapter 1 is a general introduction to malaria in Vietnam and presents the rationale for conducting the presented research;

Chapter 2 presents a detailed overview of the malaria situation in Vietnam, in terms of epidemiology, control and elimination challenges, as well as currently available diagnostic approaches;

Chapter 3 describes a new modified Semi-Nested Multiplex Malaria PCR (SnM-PCR) for the identification of the five human plasmodium species;

Chapter 4 presents the first identification of *Plasmodium knowlesi* infections in young children in Central Vietnam;

Chapter 5 reports on the marked age-dependent prevalence of symptomatic and patent infections and complexity of distribution of human Plasmodium species in Central Vietnam;

Chapter 6 describes the population genetics of *Plasmodium vivax* in four rural communities in Central Vietnam;

Chapter 7 reports on delayed parasite clearance after treatment with dihydroartemisinin-piperaquine in *Plasmodium falciparum* malaria patients in Central Vietnam;

Chapter 8 is a case report of severe malaria not responsive to artemisinin derivatives in a man returning from Angola to Vietnam;

Chapter 9 constitutes the general discussion on the findings, with limitations and implications for further improvements on malaria control and elimination in Vietnam.

References

1. Global Health Group, University of California, San Francisco: Available from: <http://globalhealthsciences.ucsf.edu/sites/default/files/content/ghg/country-briefings/Vietnam.pdf>. Last accessed 7th Dec 2015. 2012.
2. Schuftan C: A story to be shared: The successful fight against malaria in Vietnam. Available from: <http://www.eldis.org/go/home&id=14063&type=Document#.V7KMqk2KCcp>. Last assessed 7thDec, 2015. 2000.
3. Ettling MB: The control malaria in Vietnam from 1980 to 2000: What went right? Available from: http://zanran_storage.s3.amazonaws.com/www.wpro.who.int/ContentPages/20813052.pdf. Last accessed 7thDec, 2015. *World Health Organization Regional Office for the Western Pacific* 2002.
4. National Malaria Control Programme (NMCP): Annual malaria report in 2015 and malaria plan in 2016. pp. 81: National Institute of Malariology Parasitology, Entomology, Hanoi, Vietnam. Internal report 2016:81.
5. Tran Thanh Duong NDT: Malaria epidemiological stratification in Vietnam 2014. Hanoi, Vietnam: Medical publisher. ISBN: 978-604-66-0905-6; 2015. Internal report.
6. Erhart A, Thang ND, Bien TH, Tung NM, Hung NQ, Hung LX, Tuy TQ, Speybroeck N, Cong LD, Coosemans M, D'Alessandro U: Malaria epidemiology in a rural area of the Mekong Delta: a prospective community-based study. *Trop Med Int Health* 2004, 9:1081-1090.
7. Thanh PV, Hong NV, Van Van N, Van Malderen C, Obsomer V, Rosanas-Urgell A, Grietens KP, Xa NX, Bancone G, Chowwiwat N, et al: Epidemiology of forest malaria in Central Vietnam: the hidden parasite reservoir. *Malar J* 2015, 14:86.

8. National Malaria Control Programme (NMCP): Annual Malaria Report 2014. Hanoi, Vietnam: National Institute of Malariology, Parasitology and Entomology (NIMPE); 2015. Internal report.
9. Miller K: From humanitarian to economic: the changing face to Vietnamese migration Available from: <http://www.migrationpolicy.org/article/humanitarian-economic-changing-face-vietnamese-migration>. *Migration policy Insititute (MPI)* 2015, Last accessed 21thDec, 2015.
10. Obsomer V, Defourny P, Coosemans M: The *Anopheles dirus* complex: spatial distribution and environmental drivers. *Malar J* 2007, 6:26.
11. Van Bortel W, Trung HD, Hoi le X, Van Ham N, Van Chut N, Luu ND, Roelants P, Denis L, Speybroeck N, D'Alessandro U, Coosemans M: Malaria transmission and vector behaviour in a forested malaria focus in central Vietnam and the implications for vector control. *Malar J* 2010, 9:373.
12. Peeters Grietens K, Xuan XN, Van Bortel W, Duc TN, Ribera JM, Ba Nhat T, Van KP, Le Xuan H, D'Alessandro U, Erhart A: Low perception of malaria risk among the Ra-glai ethnic minority in south-central Vietnam: implications for forest malaria control. *Malar J* 2010, 9:23.
13. Hung NM: Situation of Malaria control in Vietnam during five years 2006-2010 Hanoi, Vietnam Medical publisher. Number: 240-2011/CXB/28-20/YH; 2011. Internal report.
14. White NJ: *Plasmodium knowlesi*: the fifth human malaria parasite. *Clin Infect Dis* 2008, 46:172-173.
15. Van den Eede P, Van HN, Van Overmeir C, Vythilingam I, Duc TN, Hung le X, Manh HN, Anne J, D'Alessandro U, Erhart A: Human *Plasmodium knowlesi* infections in young children in central Vietnam. *Malar J* 2009, 8:249.
16. Marchand RP, Culleton R, Maeno Y, Quang NT, Nakazawa S: Co-infections of *Plasmodium knowlesi*, *P. falciparum*, and *P. vivax* among Humans and *Anopheles dirus* Mosquitoes, Southern Vietnam. *Emerg Infect Dis* 2011, 17:1232-1239.

17. World Health Organization (WHO): Overview of malaria elimination. Available from: <http://www.who.int/malaria/areas/elimination/overview/en/>. Last accessed 7th Dec, 2015. 2015.
18. The Prime Minister of the Socialist Republic of Vietnam: Approving the national strategy for malaria prevention, control and eradication in Vietnam in the 2011-2020 period, with orientations toward 2030. Available from: http://www.chinhphu.vn/portal/page/portal/chinhphu/congdan/DuThaoVanBan?piref135_27935_135_27927_27927.mode=detail&piref135_27935_135_27927_27927.id=538. Last accessed 7th Dec DNQ-T ed. Hanoi: Ministry of Health 2011.
19. Asia Pacific Malaria Elimination Network (APMEN): Vietnam demonstrates strengthened regional commitment for a malaria-free Asia Pacific by 2030. Available from: <http://apmen.org/news/2015/3/24/media-release-vietnam-demonstrates-strengthened-regional-com.html>. Last access 18 August 2016. 2015.
20. Ministry of Health, National Malaria control and Elimination programme: National strategy for malaria control and elimination for period of 2012-2015. Available from: <http://apmen.org/apmen/Country%20Partners/Vietnam/National%2BStrategy%2Bfor%2BMalaria%2BControl%2Band%2BElimination%2B2012-2015%20Vietnam.pdf>. Last accessed 10th Oct 2016. Hanoi. Vietnam 2011.
21. Regional Artemisinin Containment Initiative Grant (RAI) Monitoring and Evaluation Plan: Addendum to Programme Management and procedural Manual (2014 -2016). Available from: http://www.raifund.org/sites/raifund.org/files/publication_docs/regional_program_evaluation_plan_for_rai_final.pdf. Last assessed 14th Oct 2016. 2014.
22. Thriemer K, Hong NV, Rosanas-Urgell A, Phuc BQ, Ha do M, Pockele E, Guetens P, Van NV, Duong TT, Amambua-Ngwa A, et al: Delayed parasite clearance after treatment with dihydroartemisinin-piperazine in *Plasmodium falciparum*

- malaria patients in central Vietnam. *Antimicrob Agents Chemother* 2014, 58:7049-7055.
23. Trung HD, Bortel WV, Sochantha T, Keokenchanh K, Briet OJ, Coosemans M: Behavioural heterogeneity of *Anopheles* species in ecologically different localities in Southeast Asia: a challenge for vector control. *Trop Med Int Health* 2005, 10:251-262.
 24. Pham VT, Hong NV, Nguyen VV, Louisa M, Baird K, Nguyen XX, Grietens KP, Le XH, Tran TD, Rosanas-Urgell A, et al: Confirmed *Plasmodium vivax* resistance to chloroquine in Central Vietnam. *Antimicrob Agents Chemother* 2015.
 25. Erhart A, Ngo DT, Phan VK, Ta TT, Van Overmeir C, Speybroeck N, Obsomer V, Le XH, Le KT, Coosemans M, D'Alessandro U: Epidemiology of forest malaria in central Vietnam: a large scale cross-sectional survey. *Malar J* 2005, 4:58.
 26. Erhart A, Thang ND, Hung NQ, Toi le V, Hung le X, Tuy TQ, Cong le D, Speybroeck N, Coosemans M, D'Alessandro U: Forest malaria in Vietnam: a challenge for control. *Am J Trop Med Hyg* 2004, 70:110-118.
 27. Tran Cong Hien HDT, Nguyen Anh Tuan: The effect on control *An. epiroticus* resistance to chemical insecticides by permament 2.0 and permament 3.0 long lasting bednets in a place of southern Vietnam. *National scientific report*. Hanoi, Vietnam Medical publisher. ISBN: 978-604-66-0950-6; 2015. Internal report.
 28. Nguyen Xuan Quang NVC, Ho Dac Thoan: Comparison of malaria transmission role of malaria vector in the residential quarters and fieldhut in some malaria endemic area of central Vietnam Hanoi, Vietnam: Medical publisher. ISBN: 978-604-66-0950-6; 2015. Internal report.
 29. Nguyen HV, van den Eede P, van Overmeir C, Thang ND, Hung le X, D'Alessandro U, Erhart A: Marked age-dependent prevalence of symptomatic and patent infections and complexity of distribution of human *Plasmodium* species in central Vietnam. *Am J Trop Med Hyg* 2012, 87:989-995.

30. Thanh NV, Toan TQ, Cowman AF, Casey GJ, Phuc BQ, Tien NT, Hung NM, Biggs BA: Monitoring for *Plasmodium falciparum* drug resistance to artemisinin and artesunate in Binh Phuoc Province, Vietnam: 1998-2009. *Malar J* 2010, 9:181.
31. World Health Organization (WHO): Emergency response to artemisinin resistance in the Greater Mekong subregion. Regional Framework for Action 2013-2015. Available from: <http://www.who.int/malaria/publications/atoz/9789241505321/en/>. Last accessed 7th Dec, 2015. 2013.
32. World Health Organization (WHO): Artemisinin and artemisinin-based combination therapy resistance. Available from: <http://apps.who.int/iris/bitstream/10665/250294/1/WHO-HTM-GMP-2016.11-eng.pdf>. Last accessed 26th Oct, 2016. 2016.
33. Bui Quang Phuc HHQ, Tran Thanh Duong: Efficacy of dihydroartemisinin plus piperazine phosphat therapy in the treatment of uncomplicated *Plasmodium falciparum* malaria in Vietnam 2014. Hanoi, Vietnam: Medical publisher. ISBN: 978-604-66-0950-6; 2015. Internal report
34. Spring MD, Lin JT, Manning JE, Vanachayangkul P, Somethy S, Bun R, Se Y, Chann S, Ittiverakul M, Sia-ngam P, et al: Dihydroartemisinin-piperazine failure associated with a triple mutant including kelch13 C580Y in Cambodia: an observational cohort study. *Lancet Infect Dis* 2015, 15:683-691.
35. National Malaria Control Programme (NMCP): Annual malaria report in 2012 Hanoi Vietnam 2013. Internal report.
36. World Health Organization (WHO): Malaria microscopy quality assurance manual version 2. Available from: http://apps.who.int/iris/bitstream/10665/204266/1/9789241549394_eng.pdf. Last access 16th June, 2016. 2016.
37. Bui Quang Phuc TTT: The sensitivity, specificity of malaria rapid diagnostic test for detecting *Plasmodium falciparum* and *Plasmodium vivax*. *Journal of malaria and Parasite diseases control* – Internal report 2015, 1:7.

38. Ta Thi Tinh NTH, Truong Thanh Ba, Nguyen Kim Thanh, Le Khanh Thuan: Evaluation and comparison the sensitivity and sensitivity of malaria rapid diagnostic test (Paracheck F test) and light microscopy in detecting *Plasmodium falciparum*. *Journal of malaria and Parasite diseases control* – Internal report 2008, 1:6.
39. Canavati SE, Lawpoolsri S, Quintero CE, Nguon C, Ly P, Pukrittayakamee S, Sintasath D, Singhasivanon P, Peeters Grietens K, Whittaker MA: Village malaria worker performance key to the elimination of artemisinin-resistant malaria: a Western Cambodia health system assessment. *Malar J* 2016, 15:282.
40. Ndyomugenyi R, Magnussen P, Lal S, Hansen K, Clarke SE: Appropriate targeting of artemisinin-based combination therapy by community health workers using malaria rapid diagnostic tests: findings from randomized trials in two contrasting areas of high and low malaria transmission in south-western Uganda. *Trop Med Int Health* 2016.
41. World Health Organization (WHO): Malaria diagnosis in low transmission setting Available from: <http://www.who.int/malaria/publications/atoz/policy-brief-diagnosis-low-transmission-settings/en/>. Last accessed 6th Dec, 2015. 2014.
42. Echeverry DF, Deason NA, Davidson J, Makuru V, Xiao H, Niedbalski J, Kern M, Russell TL, Burkot TR, Collins FH, Lobo NF: Human malaria diagnosis using a single-step direct-PCR based on the *Plasmodium* cytochrome oxidase III gene. *Malar J* 2016, 15:128.
43. Cheng Q, Cunningham J, Gatton ML: Systematic review of sub-microscopic *P. vivax* infections: prevalence and determining factors. *PLoS Negl Trop Dis* 2015, 9:e3413.
44. Escalante AA, Ferreira MU, Vinetz JM, Volkman SK, Cui L, Gamboa D, Krogstad DJ, Barry AE, Carlton JM, van Eijk AM, et al: Malaria Molecular Epidemiology: Lessons from the International Centers of Excellence for Malaria Research Network. *Am J Trop Med Hyg* 2015, 93:79-86.

45. Britton S, Cheng Q, McCarthy JS: Novel molecular diagnostic tools for malaria elimination: a review of options from the point of view of high-throughput and applicability in resource limited settings. *Malar J* 2016, 15:88.
46. Sutton PL: A call to arms: on refining *Plasmodium vivax* microsatellite marker panels for comparing global diversity. *Malar J* 2013, 12:447.
47. Murray L, Mobegi VA, Duffy CW, Assefa SA, Kwiatkowski DP, Laman E, Loua KM, Conway DJ: Microsatellite genotyping and genome-wide single nucleotide polymorphism-based indices of *Plasmodium falciparum* diversity within clinical infections. *Malar J* 2016, 15:275.
48. Jennison C, Arnott A, Tessier N, Tavul L, Koepfli C, Felger I, Siba PM, Reeder JC, Bahlo M, Mueller I, Barry AE: *Plasmodium vivax* populations are more genetically diverse and less structured than sympatric *Plasmodium falciparum* populations. *PLoS Negl Trop Dis* 2015, 9:e0003634.
49. Barry AE, Waltmann A, Koepfli C, Barnadas C, Mueller I: Uncovering the transmission dynamics of *Plasmodium vivax* using population genetics. *Pathog Glob Health* 2015, 109:142-152.
50. Arnott A, Barry AE, Reeder JC: Understanding the population genetics of *Plasmodium vivax* is essential for malaria control and elimination. *Malar J* 2012, 11:14.
51. Cattamanchi A, Kyabayinze D, Hubbard A, Rosenthal PJ, Dorsey G: Distinguishing recrudescence from reinfection in a longitudinal antimalarial drug efficacy study: comparison of results based on genotyping of msp-1, msp-2, and glurp. *Am J Trop Med Hyg* 2003, 68:133-139.
52. Felger I, Genton B, Smith T, Tanner M, Beck HP: Molecular monitoring in malaria vaccine trials. *Trends Parasitol* 2003, 19:60-63.
53. Talundzic E, Okoth SA, Congpuong K, Plucinski MM, Morton L, Goldman IF, Kachur PS, Wongsrichanalai C, Satimai W, Barnwell JW, Udhayakumar V: Selection and spread of artemisinin-resistant alleles in Thailand prior to the global artemisinin resistance containment campaign. *PLoS Pathog* 2015, 11:e1004789.

54. Neafsey DE, Juraska M, Bedford T, Benkeser D, Valim C, Griggs A, Lievens M, Abdulla S, Adjei S, Agbenyega T, et al: Genetic Diversity and Protective Efficacy of the RTS,S/AS01 Malaria Vaccine. *N Engl J Med* 2015, 373:2025-2037.
55. Preston MD, Assefa SA, Ocholla H, Sutherland CJ, Borrmann S, Nzila A, Michon P, Hien TT, Bousema T, Drakeley CJ, et al: PlasmoView: a web-based resource to visualise global *Plasmodium falciparum* genomic variation. *J Infect Dis* 2014, 209:1808-1815.

Chapter 2

Literature review on malaria

1. Malaria, a parasitic disease

Malaria is a life-threatening disease. It is caused by a protozoan parasite, which invades red blood cells and is transmitted by the female *Anopheles* mosquitos, which are present in many tropical and sub-tropical regions in Africa, Central and South America, and Asia. In order to have a complete cycle that results in malaria infection, three components are necessary: a parasite, an invertebrate host, and a vertebrate host [1].

The protozoan parasite causing malaria is from the genus *Plasmodium*, among which five species can infect humans, i.e. *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium knowlesi*, the latter being a simian species recently identified in humans in Southeast Asia [2]. The malaria life cycle involves a human host and a vector, i.e. the female *Anopheles* mosquito (Figure 1). During a blood meal, a malaria-infected mosquito inoculates sporozoites (infective parasite stage) into the human host. In less than one hour, sporozoites migrate to the liver via the bloodstream and invade liver cells (*step 2*, Fig. 1). Over 5-16 days, sporozoites grow, divide and mature into a liver schizont containing tens of thousands of merozoites per liver cell (*step 3*). Liver cells rupture and merozoites are released into the blood circulation (*4*). *Plasmodium vivax* and *P. ovale* produce a dormant liver form known as a hypnozoite that can remain dormant in the liver for weeks, months or even years, after which the parasite awakens producing a relapse (re-appearance of

parasitemia in a sporozoite-induced infection following adequate blood schizonticidal therapy) [3]. After the initial replication in the liver (*exo-erythrocytic schizogony [A]*), parasites undergo a second round of asexual multiplication in the red blood cells (*erythrocytic schizogony [B]*). Merozoites invade red blood cells (5) and develop from immature (ring stage) to mature trophozoites, and into schizonts, which will then rupture and release new merozoites (6) into the blood circulation, that will in turn infect new red blood cells. Some merozoite-infected blood cells will develop into sexual erythrocytic stages, called male and female gametocytes (7). When a mosquito bites an infected human (8), it ingests the gametocytes, which develop into mature sexual forms called gametes. In the mosquito's stomach (*sporogonic cycle [C]*), male (micro) and female (macro) gametes fuse to form a diploid zygote (9), which becomes motile and elongated (ookinetes) (10) and penetrates the midgut wall of the mosquito where it develops into an oocyst (11). After 8-15 days growth, oocysts rupture releasing sporozoites (12) which migrate and invade the mosquito's salivary glands where they are stored until the next blood meal [4-6].

The clinical manifestations associated with malaria are caused by the asexual blood stage of the parasites and may result in a wide variety of symptoms, ranging from absent or very mild symptoms, *i.e.* asymptomatic infections, to severe disease, and even death. The typical clinical symptoms of an uncomplicated malaria infection are periodic fever, headache, weakness, aching muscles, sweats and chills, vomiting, and diarrhea. The most typical symptom, *i.e.* fever, is caused by numerous known and unknown waste

substances such as hemozoin pigment and other toxic factors, which accumulate in the infected erythrocytes and are released into the bloodstream during erythrocyte rupture and merozoite release. These parasitic molecules stimulate macrophages and other cells that produce cytokines that act on the hypothalamus producing fever and chills. Severe malaria is mostly due to *P. falciparum* infections and it frequently occurs in immunologically naive people, including children under five. Biological characteristics of *P. falciparum* associated with severe malaria are cytoadherence, erythrocyte destruction and rosetting [7]. Clinical manifestations include cerebral malaria, severe anemia, hemoglobinuria, acute respiratory distress syndrome, abnormalities in blood coagulation, acute kidney failure, metabolic acidosis, hypoglycaemia, etc. Severe malaria is also reported with *P. vivax* and *P. knowlesi* [8, 9] but the severity of symptoms varies widely between different geographical areas [9]. In general, malaria is a curable disease if diagnosed and treated promptly with effective drugs.

Today, malaria is found mainly in sub-Saharan Africa, the Amazonia region and Southeast Asia. It affects half of the world's population, causing approximately 250 million clinical cases and 500,000 deaths each year [10]. Within endemic countries, the poorest and most marginalized communities are the most severely affected.

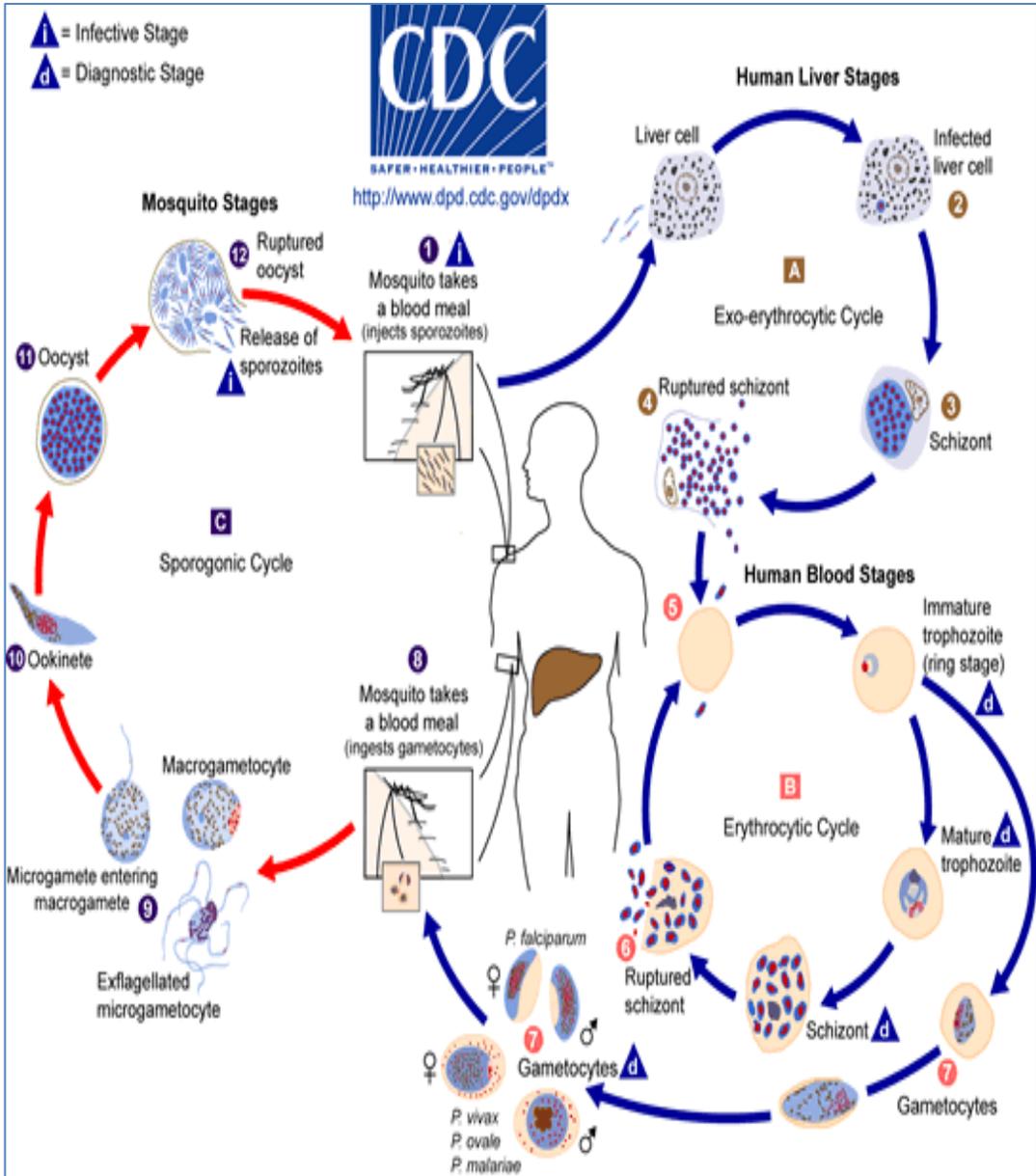


Figure 1. Life cycle of the malaria parasite

(<http://www.cdc.gov/malaria/about/biology/index.html>)

2. The global burden of malaria

2.1. Malaria in the world

The remarkable increase of financing and strengthening of NMCPs overall has resulted in a large reduction in the number of malaria cases and deaths in the last decade. Between 2000 and 2015, the annual number of malaria cases reduced from 262 million to 214 million, and malaria deaths from 839, 000 to 438,000, representing a reduction of 37% and 60%, respectively (Figure 2A) [10]. The most remarkable reduction in mortality rate, i.e. by 65%, was observed among children under five years of age. Among the 106 malaria endemic countries reported in 2000, more than half (n=57) reduced malaria case incidence by more than 75%, while in 18 countries malaria incidence was reduced by 50-75% (Figure 2B) [11]. As a result, malaria was eliminated from the United Arab Emirates in 2007, Morocco and Turkmenistan in 2010, Armenia in 2011 [10] and Sri Lanka in 2016 [12], and an increasing number of countries are moving toward malaria elimination.

Despite the global control efforts and progress in reducing malaria morbidity and mortality over the past 15 years, malaria remains a heavy burden in some highly endemic and vulnerable countries. At present, 89% of all malaria cases and 91% of malaria deaths occur in sub-Saharan Africa, followed at distance by the Southeast Asia (SEA) region, home to 10% of cases and 7% of deaths [10].

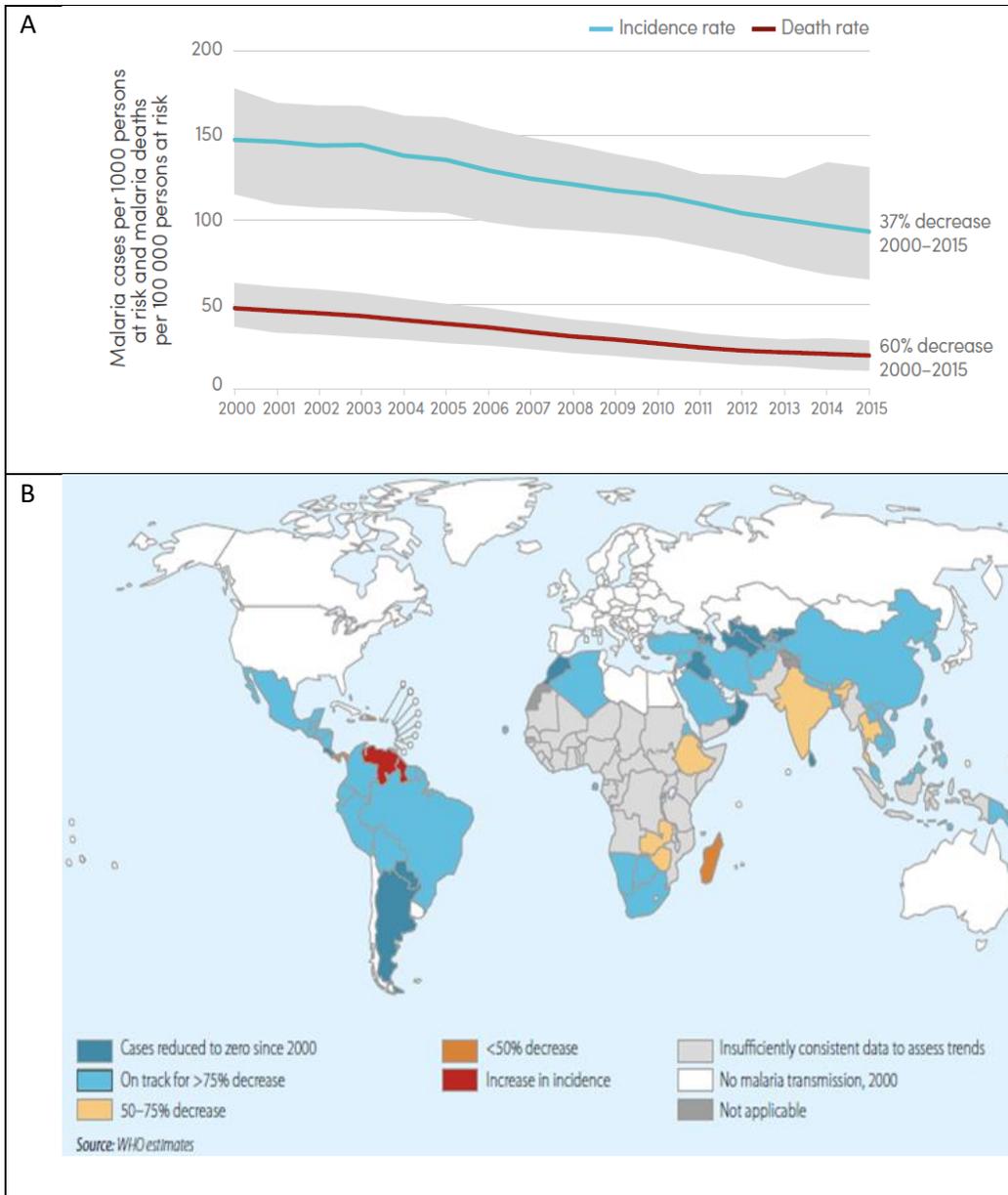


Figure 2. Malaria in the world (2000 – 2015)

- A) Estimated changes in global malaria incidence and mortality rate between 2000 to 2015 [10].
- B) Projected changes in malaria incidence rates by 2015 among 106 countries endemic for malaria in 2000 [11].

2.2. Malaria in Mekong sub-region

In 2014, 20 million malaria cases and 812 deaths occurred in the SEA region [10]. Malaria incidence in the region varies greatly with some countries such as China and Vietnam being on track for malaria elimination while others such Burma are just starting to scale up malaria control activities [13]. In 2015, 74% of all *P. vivax* cases worldwide occurred in the SEA Region. Within SEA, the Greater Mekong sub-region (GMS) comprises six countries, *i.e.* Thailand, Laos, Cambodia, Myanmar, Vietnam, and the Yunan province of China, concentrating the highest malaria burden in the area. Malaria in the GMS is characterized by transmission concentrated in forested areas, which are the habitat of the main malaria vectors *An. minimus* and *An. dirus* [14]. These species have an outdoor and early biting behavior [15] that challenges standard vector control measures based on IRS and insecticide-treated bed-nets. In addition, multiple secondary vector species such as *An. sudaicus*, *An. sinensis*, *An. maculatus*, *An. philippinensis* etc., further contribute to malaria transmission in those areas. Remote forested areas are usually home to local ethnic minorities and migrant workers which represent two highly vulnerable groups due their regular forest activities, low socio-economic and education level, and for migrant workers, often with poor access to the local health system and/or preventive measures [16]. The situation is further complicated by the complexity of the parasite reservoir (high frequency of sub-microscopic infections with multiple species [10, 17-19], and the development and spread of drug resistant parasites [20, 21].

Even though the malaria situation in the GMS has greatly improved during the past 15 years, with a total reduction in the mortality rate of 90% and a reduction of malaria incidence of more than 50%, this reduction has not been homogeneous, and in five of the six GMS countries (with the exception of China) the disease remains a public health problem. In Myanmar and Laos, malaria morbidity in 2014 accounted for 152,195 (2.8/1,000 pop) and 48,701 cases (7.1/1,000 pop), respectively. In the same year China had the lowest malaria mortality rate (0.0002/10,000pop), while Myanmar carried the highest rate of death (0.02/10,000pop) [10].

2.3. Malaria in Vietnam

The evolution of malaria morbidity and mortality since 2006 is shown in Figure 3. From 2000 to 2015, malaria morbidity and morbidity in Vietnam has been reduced by more than 93.4% and 97.9%, respectively [22, 23], prompting the country to officially engage in malaria elimination with the goal of having a malaria-free country by 2030 [24].

According to the latest malaria epidemiology stratification done by the NMCP in 2014, an estimated 11.7 million people (12.5% of the total population) are still living in areas at risk of malaria; most of the high risk areas being situated along the border with Laos and Cambodia [25]. In 2015, a total of 9,331 confirmed malaria cases and only three deaths were reported nationwide, resulting in an overall annual incidence of 0.101/1,000 population. However, this figure peaked at 1.59/1,000 population in the high-risk area of Central Vietnam. Indeed, more than 80% of the malaria cases reported in 2015 occurred in only 10 provinces in Central Vietnam [23], among which Gia Lai and

Binh Phuoc province accounted respectively, for 23.7% and 19.3% (n=2,245 and 1,799) of the total cases and an incidence rate of 1.6/1,000pop and 1.9/1,000pop, followed by Ninh Thuan (0.9/1,000pop) and Dak Nong province (0.7/1,000pop).

The main malaria species in Vietnam are *P. falciparum* and *P. viva*, *P. malariae* and *P. ovale* are rarely observed by LM [22, 26]. In 2009, *P. knowlesi* was identified for the first time in individuals from south-central province of Ninh Thuan [27].

A total of 59 different *Anopheles* species have been identified in Vietnam, among which 15 are involved in malaria transmission [15, 28], with *An. minimus s.l.* being the main vector in northern Vietnam, *An. dirus sensu stricto* in Central Vietnam (from Quang Tri to Binh Thuan), and *An. epiroticus* in the Mekong delta [15].

Currently, the first-line treatment for *P. falciparum* mono- or mixed infections consists of a three-day course of DHA-PPQ followed by a single dose of primaquine (PQ) at 0.5mg/kg body weight (bw) to clear gametocytes [29]. Chloroquine (CQ, 3 days) remains the first-line treatment for *P. vivax* infections, combined with 14 days of primaquine (0.25mg/kg bw) as anti-relapse treatment. The second-line treatment consists of Quinine and Doxycycline for seven days for *P. falciparum* infections and DHA-PPQ for three days for *P. vivax* infections. Pregnant women and children under 8 years of age receive Quinine and Clindamycin (7 days).

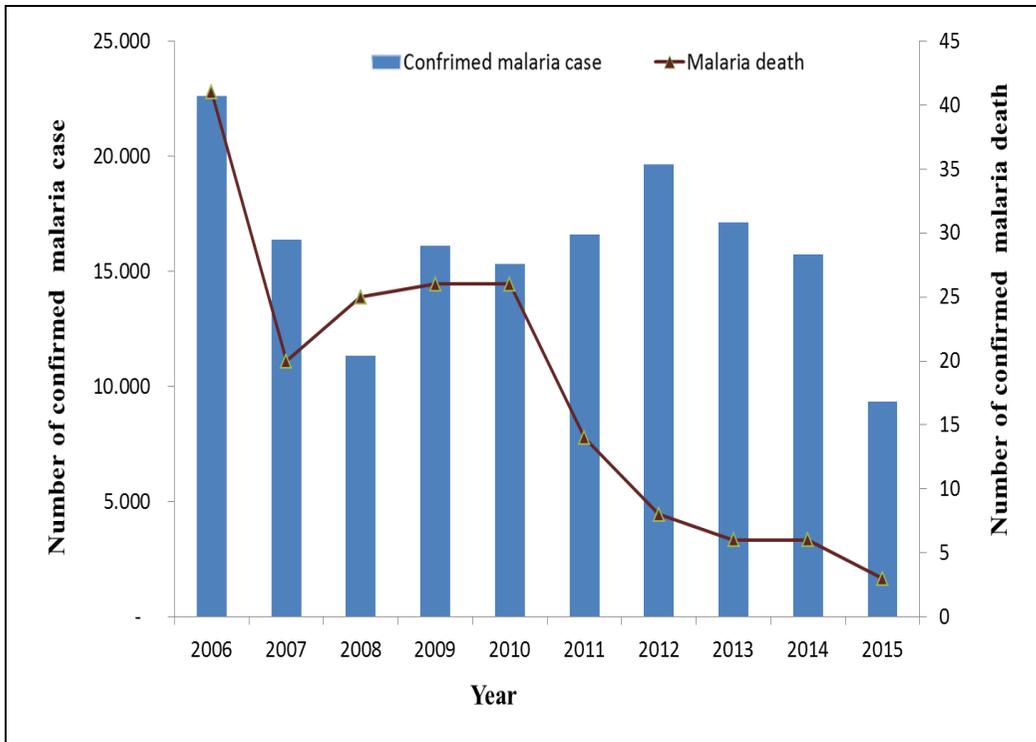


Figure 3 : Evolution of malaria morbidity and mortality from 2006 to 2015 [22, 23]

3. Malaria control and elimination strategies

3.1. Definitions

Malaria control is defined as the reduction of malaria morbidity and mortality to a level at which it is no longer a public health problem, through deliberate efforts using known effective prevention tools such as vector control, chemoprophylaxis and case management tools [30]. Vector control measures which include IRS and insecticide-treated nets (ITNs) are the most powerful and broadly applied interventions to reduce vector daily survival rate, and are intended to protect individuals against infective

mosquito bites and reduce the intensity of local malaria transmission at the community level [31]. Early diagnosis and prompt treatment with effective antimalaria drugs is the core pillar to reduce mortality and morbidity. ACTs are currently the only recommended first-line treatment against *P. falciparum* infections.

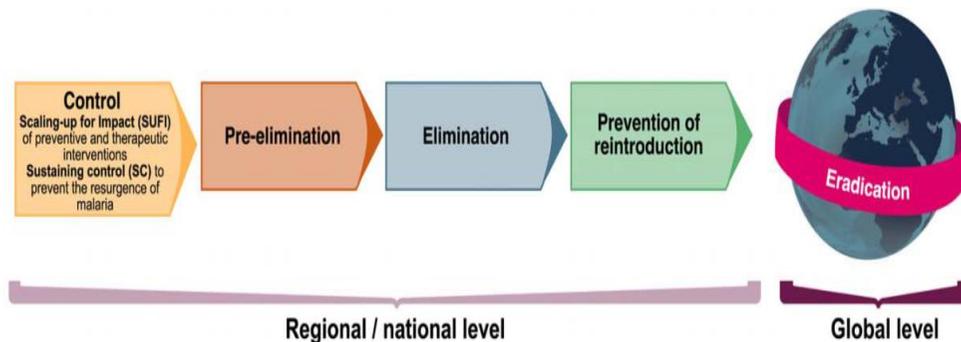


Figure 4. Global malaria elimination continuum [32].

Malaria elimination involves the interruption of local mosquito-borne malaria transmission, *i.e.* the reduction to zero of the incidence of malaria infections in a defined geographical area [33]. From a country perspective, elimination of malaria is the ultimate goal of malaria control. The path towards malaria-free status is characterized by four distinct programme phases: control, pre-elimination, elimination and prevention of reintroduction (Figure 4 & Box1) [10, 33]. Each phase is defined by a set of specific programme interventions required for prevention, treatment, surveillance, monitoring and evaluation, and health system strengthening.

Box 1. Definitions for stages towards malaria elimination [10]

Malaria control programmes are implemented in countries for reducing the malaria disease burden (incidence, prevalence, morbidity, and mortality rates) to a level at which it is no longer a public health problem. Continued intervention measures are required to maintain the reduction.

Pre-elimination programmes are implemented in countries to reorient their NMCP between the sustained control and elimination stages. During this period, high coverage with good-quality laboratory and clinical services should be reached, reporting and surveillance should be reinforced, followed by other programme adjustments to halt transmission nationwide.

Malaria elimination is the complete interruption of the local mosquito-borne malaria transmission, i.e., reduction to zero of the incidence of infection caused by human malaria parasites in a defined geographical area as a result of deliberate efforts. Continued measures are required to prevent reestablishment of transmission.

Prevention of reintroduction programmes are implemented in countries that have either recently achieved zero cases and aim to maintain the situation, or in countries that are generally considered non-endemic, having been malaria-free for well over a decade, that have experienced recent outbreaks of locally acquired malaria subsequent to importation of parasites. The main activity is vigilance (surveillance and response) by the general health services, which can be combined with vector control and other interventions to reduce receptivity in vulnerable areas.

Malaria eradication is the permanent reduction to zero of the worldwide incidence of infection caused by a particular malaria parasite species as result of deliberate efforts. Intervention measures are no longer required once eradication has been achieved.

3.2. History of malaria control

During the last century, immense effort was made to control and eliminate malaria, with limited impact [34]. In 1955, the WHO launched the Global Malaria Eradication Programme to eliminate malaria in all parts of the world except Saharan Africa. The strategies used focused on vector control, active detection and treatment [35]. However, a drastic increase in malaria was observed between the 1970s and 1980s due to multiple factors: a decrease in malaria preventive measures, reduction of immunity levels in some populations, development and spread of chloroquine resistant parasites, and the fact that the same strategies were applied everywhere despite differences in parasite and mosquito species. By 1972, the Global Malaria Eradication programme was officially abandoned [36], and by 1978, malaria was eliminated from 37 countries and two continents, i.e. Europe and Australia [35, 37].

At the beginning of the 1990s, annual malaria estimates were about 300-500 million clinical cases and 1.5-2.7 million deaths [38]. The WHO Global Malaria Control Strategy was launched in 1992 [39] to promote a rational and selective use of tools (focus on primary health care, flexible and decentralized programmes) to combat malaria, mainly through early diagnosis and effective treatment together with selective and sustainable vector control measures [38]. The Roll Back Malaria Partnership (RBM) was formed in 1998, with the main aim to reduce malaria cases through the widespread application of existing health measures and new technologies. In September 2008, the RBM launched the Global Malaria Action plan

focused on long-term malaria control programmes incorporating multiple activities and embracing multiple interventions, disciplines, approaches and organizations to control the mosquito vector and provide preventive therapies and/or effective curative treatment [37, 40]. The goal was to achieve 50% reduction of malaria cases by 2000 and less than 500,000 malaria deaths by 2010. However, malaria prevalence only dropped 26% by 2011[41].

3.3. Elimination goals and strategies in the GMS

The WHO New Global Strategy 2016-2030 aims at reducing malaria case incidence and mortality by 90% in 2030 compared to 2015, eliminating malaria from at least 35 countries with ongoing malaria transmission in 2015, and preventing re-establishment of malaria in all malaria-free countries by 2030 [42].

Existing strategies for malaria control such as vector control measures (LLINs, LLIHNs, IRS), early diagnosis and effective treatment with ACTs, surveillance (ACD, Passive case detection (PCD)), and mass screening have been quite successful in reducing malaria in the GMS, as reflected by a steady decline in annual malaria incidence and deaths in the region [10]. However, as malaria transmission decreased to very low levels, new challenges to achieve malaria elimination emerged, *i.e.*, geographical heterogeneity in malaria transmission [43-45] with high levels of transmission occurring along international borders and forested areas [46], and high diversity in vector systems with dramatic differences in their ecology, biting behavior, and vectorial capacity [45].

Furthermore, the GMS, and more specifically the western Cambodian border, is known as the “cradle” of *P. falciparum* resistance to different antimalarial drugs. Chloroquine (CQ) resistance emerged in the late 1950s, followed by resistance to sulfadoxin–pyrimethamine (SP) (in 1967) and mefloquine (MQ) (in 1985); eventually artemisinin (AS) resistance emerged in the mid-2000s [13, 21, 47]. The latter represents a serious threat to the global malaria control efforts as ACTs remain currently the most efficacious antimalarial commercially available. The Mekong Roll Back Malaria Initiative was launched in 1999 with the aim of reducing malaria mortality in the GMS by 50% by 2010 (compared to 1998), and halting the progress of multi drug resistance through the strengthening of NMCPs, health services, and multi-sectorial approaches with different partners [48]. Artemisinin resistance was first reported in western Cambodia (Pailin province) in 2009 [49]. The Global Plan for Artemisinin Resistance Containment (GPARC) and the Emergency Response to Artemisinin Resistance (ERAR) were launched in 2011 and 2013, respectively [50, 51], and by 2015, artemisinin resistance was reported in five GMS countries, *i.e.* Cambodia, Vietnam, Laos, Thailand, and Myanmar [52].

Due to the rapid spread of artemisinin resistant *P. falciparum* parasites, the elimination of this species has become a high priority in the GMS. Thus, WHO has established a Strategy for Malaria Elimination in the Greater Mekong sub-region (2015-2030) to rapidly interrupt malaria transmission in drug resistance areas with the aim of eliminating *P. falciparum* malaria by 2025, and all malaria species in all

countries of the GMS by 2030 [10, 46]. Even if reduction is attained, maintaining zero cases achievement is difficult due to population movements between endemic and non-endemic areas, with the subsequent threat of re-establishment of malaria transmission in areas where it had already been eliminated. Currently, there is no guideline on how to quantitatively assess, based on key epidemiological indicators, the feasibility of achieving and maintaining elimination in a given area [18].

3.4. Current malaria control and elimination activities in Vietnam

In 2014, the Vietnamese NMCP defined a new malaria epidemiological stratification map based on confirmed malaria cases reported from 2009 to 2013 (Figure 1, Chapter 1) [25]. This new stratification classified malaria endemic areas into five categories according to the level of local malaria transmission in the past five years, *i.e.* from no local transmission to areas where annual incidence exceeded 5 cases/1,000pop/year. Moreover, each commune was also allocated a score combining the past five years' annual incidence rate (ARI) together with other criteria, as follows: *i)* ARI < 1 case/1,000 pop/year = 5 points; *ii)* ARI: 1- < 5 cases/1,000pop/year = 10 points; *iii)* ARI ≥ 5 cases/1,000pop/year = 15 points; *iv)* evidence of drug-resistant malaria parasites = 10 points; *v)* presence of main malaria vectors (*An. minimus*, *An. dirus s.s.*, *An. epiroticus*) = 1 point; *vi)* extremely difficult economic communes and/or border communes = 1 point; *vii)* weak commune health care network = 1 point; *viii)* vector resistance to insecticides = 1 point; *ix)* residential mobility = 1 point [25]. The map shows that malaria is mainly concentrated in the Central Highlands and Southeast provinces, as well as in border

areas with China, Laos and Cambodia [25]. The main goal of the NMCP is to continue to roll back malaria with the specific objectives to reduce malaria morbidity below 0.15/1,000 population, mortality below 0.02/100,000 population, and to eliminate malaria from at least 40 provinces by 2020 and from the whole country by 2030 [24].

Following this new classification system, different interventions are recommended (summarized in Table 1):

- Early diagnosis and prompt treatment during both PCD, at the commune and village levels, as well as during regular ACD campaigns, is carried out by hamlet health workers (HHWs) and mobile teams from the district or provincial malaria centers. Currently, 80% of all antimalarial treatments are administered at the peripheral (commune + village) level, and 70% of malaria cases are diagnosed in health facilities (all levels) within the first 24 hours of the onset of symptoms [26].
- Vector control activities, including ITNs/LLINs distribution and IRS, are carried out annually, or twice a year in the remote and epidemic-prone areas before and during the malaria season. Long lasting insecticidal nets and hammocks nets are also provided free of charge for most ethnic minority groups living in endemic areas. The sensitivity of mosquitoes to insecticides is regularly monitored by NIMPE, Hanoi.
- A malaria surveillance system based on the continuous monitoring of epidemiological parameters (prevalence of infections and parasite species, incidence of clinical and severe cases, etc), treatment policy, and early detection of outbreaks has been set up in all strata, and weekly reports are sent from each province to the central level based

on an electronic system and hotlines. This allows the NMCP to assess the malaria situation in real time and to respond in a timely manner with adequate interventions.

- Health education is implemented in all strata (not shown in Table 1), education and communication messages being translated and adapted to the numerous local minorities living in the malaria endemic areas using different support measures such as posters, brochures, calendars, books, etc. Moreover, information on malaria prevention is provided nation-wide by different mass media (TV, radio, Internet). The World Malaria Day is organized annually in malaria endemic areas to raise community awareness [53].
- Research activities focus on current challenges to malaria elimination perspectives, such as the difficulty of controlling *P. vivax* malaria, the emergence of drug resistant parasites, the asymptomatic and sub-microscopic human parasite reservoir, and uncontrolled population movements both within (between endemic and malaria free provinces) and across international borders [25].
- Planning and evaluating malaria elimination strategies relies on the accurate measurement of the prevalence and incidence of malaria infections in the population. Therefore, Vietnam needs more sensitive tools and approaches to detect and eliminate asymptomatic and sub-microscopic infections, which form the largest part of the malaria reservoir [54] but are not detected with the currently used diagnostic tools (i.e., LM and RDT).

Table 1. Stratification of malaria endemicity (2014) with main control strategies

CHARACTERISTICS	AREAS	INTERVENTIONS FOCUS
No local malaria transmission	Malaria free areas (Prevention of re-introduction)	Early diagnosis & prompt treatment (ED&PT) of imported malaria cases.
Former malaria endemic areas without local transmission during last 5 years.	Risk of malaria resurgence (Prevention of re-introduction)	<ul style="list-style-type: none"> - ED&PT of imported malaria cases. - Antimalaria drugs for self-treatment in people going to malaria endemic areas (stand by treatment); - Strict management and investigation of imported malaria cases, regular surveillance (PCD + ACD); identification and monitoring of migrants; - Provision of ITNs for people going to malaria endemic areas.
5– 8 points	Low malaria endemic area (malaria elimination phase)	<ul style="list-style-type: none"> - ED&PT of imported malaria cases; standby treatment; - ACD of malaria cases, investigation of malaria infections (GIS) strengthen microscopic points, involvement of private sector in malaria control, monitoring of antimalaria drugs selling; monitoring of residential mobility; - ITNs in the bordering areas with moderate and high risk of malaria, IRS during malaria outbreaks; - Collaboration with other ministries and sectors.
9- 13 points	Moderate malaria endemic area (pre-	<ul style="list-style-type: none"> - ED by LM or RDTs for 100% suspected malaria cases, prompt and effective

	<p>malaria elimination phase)</p>	<p>treatment + primaquine for <i>P. f</i> gametocytes and anti-relapse of <i>P. v</i> and <i>P. o</i>;</p> <ul style="list-style-type: none"> - Regular malaria surveillance; monitoring antimalaria drug resistance; GIS for malaria infections and vectors, consolidate peripheral health services (microscopy points), involve private sector in malaria diagnosis, treatment, control and the selling of antimalaria drugs; - Annual LLINs or ITNs distribution for local people, IRS where <80% of people sleep under bed-nets and in epidemic prone areas; - Collaboration with other ministries and sectors, strengthening collaboration with military in malaria control.
14 -30 points	<p>High malaria endemic area (control and consolidation phase)</p>	<ul style="list-style-type: none"> - Mobilization to sleep under ITNs especially in the fields, or forest; - ED by LM or RDTs for 100% suspected malaria cases, prompt and effective treatment, primaquine for <i>P. f</i> gametocytes and anti-relapse of <i>P. v</i> and <i>P. o</i>; In communes with artemisinin resistance, malaria treatment is direct supervised (DOT); - Monitoring malaria drug resistance; GIS mapping for malaria infections and vector; consolidate peripheral health services (microscopy points), involve private sector

		<p>in malaria diagnosis, treatment, control and the selling of antimalaria drugs.</p> <ul style="list-style-type: none"> - ITNs or LLINs annually for local people; priority IRS and ITNs in epidemic prone areas; Implement IRS where <80% of people sleep under bed-nets. In high risk areas, IRS and ITNs can be done 2 times/year if ITN coverage<80%. Multiple collaborations with other ministries, and military; promote community-based malaria prevention.
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ITNs=insecticide treated bed-nets; LLIN= long lasting insecticidal nets; *P. f*=*P. falciparum*; *P. v*= *P. vivax*; *P. o*=*P. ovale*; ACD=active case detection; PCD=passive case detection

4. Antimalarial drug resistance

4.1. *P. falciparum* resistance to artemisinin

All ACTs combine a fast-acting ART drug such as AS or DHA-PPQ which can reduce the parasite biomass by four orders of magnitude every 48 hours [55] with a longer-lasting antimalarial partner drug, such as lumefantrine (LF), amodiaquine (AQ), MQ, PPQ or sulphadoxine-pyrimetamine (SP), able to eliminate any remaining parasite from the blood.

The first reports of higher recrudescence rates (4.2%) of *P. falciparum* malaria after treatment with artemisinin-based combination treatments (ACTs) emerged from observational data collected in Cambodia since 2004 [56], but it was not clear initially whether these higher failure rates resulted from resistance to artemisinins (ART), their

partner drugs, or unusual host or pharmacokinetic factors [57]. Four years later, resistance to ART, defined as increased parasite clearance after treatment with ACTs or ART-monotherapy was reported in western Cambodia [49, 58]. Since then, it has also been detected in Myanmar, Laos, Thailand, and Vietnam [59-62], though ACT's cure rates were still more than 90% [63, 64]. The proportion of patients with a positive parasitemia on day three has traditionally been the best indicator to measure *P. falciparum* sensitivity to ART in therapeutic efficacy studies.

In 2010, an African lab strain (F32-ART) that can survive extremely high doses of ART (up to 7,000-fold the initial IC₅₀) was obtained through long-term (three years) artemisinin pressure *in vitro* [65]. The study demonstrated that prolonged drug pressure with ART could induce a sub-population of ring stages into developmental arrest, proposed mechanism of ART resistance. In 2014, SNPs in the Kelch propeller domain of the K13 gene (K13) were identified as ART resistant marker using whole genome sequencing (WGS) of an artemisinin resistant parasite line from Africa selected under escalating ART pressure and 49 clinical parasite isolates from Cambodia resistant to ART and culture-adapted. These K13 mutations were associated with an increased proportion of early-rings parasites that survived 700 nM DHA exposure or increased parasite clearance half-life (>5 hours) [66, 67]. The correlation between K13 mutations and ART resistant strains was also shown in *ex vivo* ring stage survival assays (RSAs), consisting of a short-term exposure to high concentration of ART which proved to better discriminate between drug resistant and sensitive strains [66, 68]. After removal of K13 mutations in isolates

from Cambodia using zinc-finger nucleases, parasite survival rates decreased from 13-49% to 0.3-2.4%; in contrast, after insertion of the R539T, M476I and C580Y K13 mutations, survival rates increased from 0.6% to 2-29% [69].

Since September 2015, the WHO incorporated K13 mutations in its working definition of ART resistance as follows: *i) suspected artemisinin resistance* is defined as high prevalence of the delayed parasite clearance phenotype ($\geq 10\%$ of patients with persistent parasitemia by microscopy on day 3 after treatment with ACT or AS monotherapy), OR high prevalence of K13 mutants ($\geq 5\%$ of patients carrying K13 resistance-associated mutations); *ii) confirmed artemisinin resistance* is defined as delayed parasite clearance AND K13 resistance associated mutation in a single patient [52].

Currently, a total of 186 K13 propeller mutations, including 108 non-synonymous mutations [52, 70], have been reported, among which four Asian mutations (Y493H, R539T, I543T, C580Y) were “confirmed mutations” with *in vivo* and *in vitro* data” [67, 69, 71, 72], while another 13 were “associated mutations” (*i.e.*, correlating with delayed parasite clearance) [52, 70, 72-75]. In Vietnam, Cambodia, Thailand and Laos, the four above-mentioned confirmed K13 mutations are the most frequent ones, while four other associated mutations (F446I, F446L, N458Y, P574L and R561H) are common in Myanmar and China (Figure 5) [52, 76].

Despite the steady decline in parasite clearance rates along the GMS, ACTs remained largely effective. However, in 2015, studies in Cambodia reported an increase in

treatment failure rates following DHA-PPQ therapy reaching 54% associated with high prevalence of K13 mutations and a significant increase in PPQ IC₅₀s [77]. Yet another study reported the association of K13 mutation with decreased copy numbers of the *Pfmdr1* gene, and that K13 mutation together with an *ex vivo* piperazine survival rate (RSA) $\geq 10\%$ was associated with a 32-fold higher risk of recrudescence [78]. The results were interpreted as being indicative of the frightening spread of resistance for both DHA and PPQ drugs [78]. In 2016, by investigating the whole genome sequences and piperazine susceptibilities of 297 *P. falciparum* clinical isolates from Cambodia, *exo-E415G* SNP and *plasmepsin 2–3* amplification were identified as markers of decreased piperazine susceptibility *in vitro* and DHA-PPQ treatment failure in Cambodia [79]. This finding might help to monitor the spread and understand the mechanism of piperazine resistance in GMS.

In Africa, K13 mutants were identified at a very low prevalence (<5%) [80, 81], and the most frequent allele (A578S) has not been correlated with delayed parasite clearance [52]. Among the 25 K13 mutant alleles identified in Africa, 21 were novel alleles while 4 had been identified previously in the Mekong Sub Region [82]. Despite the absence of evidence on ART resistance in sub-Saharan Africa, serious concerns were raised on the spread of ART resistant strains following the increasing numbers of Asian migrants working in Africa [83], as well as the presence of K13-mutant alleles previously associated with the slow clearance of parasites in Asia [59, 84]. This situation is worryingly reminiscent of previous emergence and spread of resistance to chloroquine,

sulfadoxine–pyrimethamine and mefloquine [13, 21, 47]. Artemisinin resistance is a major threat to health security, with the most severe potential effects in sub-Saharan Africa, where the disease burden is highest and systems for monitoring and containment of resistance are inadequate [85].

4.2. Chloroquine resistance

P. falciparum developed resistance to chloroquine (CQR) in the 1950s [86], starting in Colombia and at the Cambodia-Thailand border, spreading rapidly through the continents to reach East Africa in the 1970s [86]. After only one decade, *P. falciparum* CQR was reported in all sub-Saharan African countries [86]. On the other hand, the first reports on *P. vivax* CQR originated in Papua New Guinea in 1989 [87] and were rapidly followed by reports in Indonesia [88], and other countries in Southeast Asia [89]. Currently, evidence of *P. vivax* CQR parasites with diverse degrees of resistance have been described in several *vivax* endemic areas: Pacific (Indonesia, Philippines), Southeast Asia (Myanmar, India, Vietnam), South America (Colombia, Guyana), and the Middle East (Turkey) [20, 90].

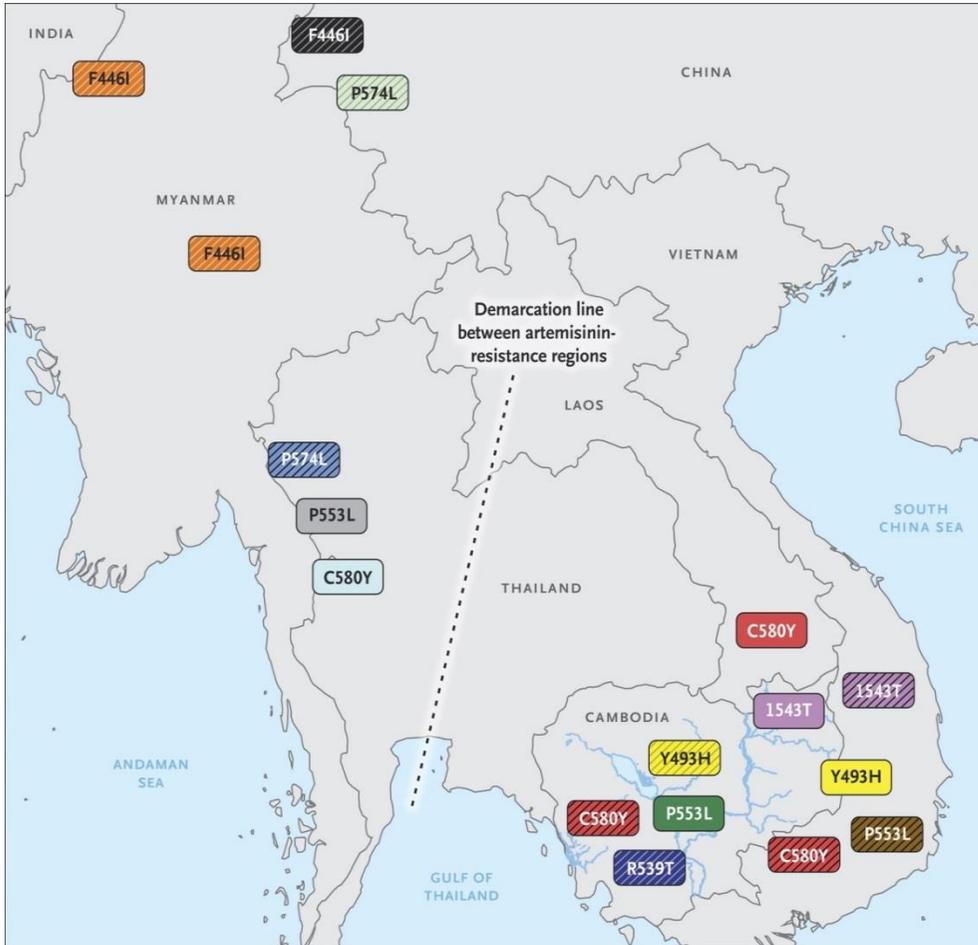


Figure 5. Distribution of the flanking haplotypes of the C580Y, Y493H, R539T, I543T, P553L, P574L, and F446I non-synonymous mutations in K13 in Cambodia, Vietnam, Laos, western Thailand, Myanmar, and China [70].

The haplotype groups are shown in distinct colors. Similarly colored boxes indicate shared haplotypes, and hatched boxes indicate the presence of additional country-specific haplotypes.

Currently, the *P. vivax* orthologous genes (*pvmdr1* and *pvcr1*) of the *pfmdr1* and *pfcr1* genes (associated with *P. falciparum* CQR) are considered as the putative markers of CQR in *P. vivax* (PvCQR) [91-93]. Indeed, the level of *pvcr1* transcription has been reported to increase 22-fold in severe vivax, compared to uncomplicated malaria patients [94]. In addition, in a study conducted between 2003 and 2007, *pvmdr1* was found in all *P. vivax* patients in Papua, Indonesia, where CQ resistance is highly prevalent [95]. While the Y976F mutation in *pvmdr1* has been strongly associated with increased CQ IC₅₀ [95-97], no associations have been found so far between point mutations in the *pvmdr1* and the clinical response of vivax malaria to chloroquine [98]. Therefore, our knowledge about the mechanisms and molecular markers of CQ resistance in *P. vivax* remains limited mainly due to the technical challenge in maintaining this parasite under *in vitro* conditions [99].

Therefore, therapeutic efficacy studies remain a central tool for monitoring the efficacy of nationally recommended antimalarial treatments in all endemic countries, and genetic tools help to identify drug resistance markers even among populations with asymptomatic malaria.

4.3. Antimalarial drug resistance in Vietnam

P. falciparum resistance to CQ was first reported in 1961 in Central Vietnam [100], in 1985 in the northern provinces, and by 1994 CQR was prevalent in the whole country [101]. At the same time, *P. falciparum* resistance to sulfadoxine-pyrimethamine, quinine, and MQ were also reported [52, 101]. Therefore, artemisinin derivatives

started to be used as a first-line antimalarial therapy as early as 1995, first as monotherapy (for seven days), and from 1999 as combination therapy (DHA-PPQ co-formulated with primaquine and trimethoprim also known as “CV8”) [55, 102]. Since 2003, DHA-PPQ is recommended as the first-line antimalarial treatment in Vietnam [103]. The NMCP, with the support of the WHO and the Global Fund (GF), has been monitoring ACTs resistance with TES in several sentinel sites across the central and southern provinces (Figure 6). From 2006 to 2015, the collected data showed that adequate clinical parasitological rates (ACPRs) for both 7-day AS and 3-day DHA-PPQ regimens remained above 90%. However, the number of provinces with reasonable evidence of artemisinin resistance (also called “Tier I area”) increased rapidly from one in 2009 (Binh Phuoc) to five in 2015 (Binh Phuoc, Khanh Hoa, Quang Nam, Kom Tum and Gia Lai). The day-3 positivity rate after treatment also followed a steady increase (e.g. in Binh Phuoc, the D3 positivity rate rose from 15.2% in 2010 to 22.8% in 2012, to 36% in 2014 and to 57% in 2015; in Gia Lai province, this increased from 11.2% in 2010 to 22.8% in 2012, to 24.6% in 2014 and reached 57% in 2015 [104-106].

PvCQR was first reported in 2002 in southern Vietnam [107], though this was not confirmed with CQ blood level measurements. Since then, *P. vivax* sensitivity to CQ was regularly monitored and was considered sensitive since 100% ACPR was reported between 2006 and 2011 (total 350 patients) within the six sentinel sites in central Vietnam [104]. In 2015, *PvCQR* was confirmed for the first time with blood level measurements at the time of recurrence (> MIC of 100ng/ml) in Quang Nam province,

in the same site where *P. falciparum* resistance to artemisinin was reported one year earlier [108, 109]. Retrospective data (2006-2015) on CQ and ACTs efficacy for the treatment of uncomplicated *P. vivax* and *P. falciparum* malaria in Vietnam is shown in Figure 6.

Factors that have fuelled the development of antimalarial drug resistance in Vietnam include poor use of primaquine, inadequate treatment regimens, self-treatment, poor patient adherence to prescribed antimalarial regimens, and the widespread availability of oral artemisinin-based monotherapies and substandard forms of the drug [110-112]. Moreover, the spread of resistant *Plasmodium* strains to different provinces in the North or in the Mekong delta is potentially facilitated by the continuous population movements towards and from Central Vietnam, where numerous agriculture-based economic activities attract more and more workers from within and across international borders [50, 113]. Therefore, artemisinin resistance represents a real threat to malaria elimination strategies in Vietnam, and as in other countries in the GMS, poses a major health security risk as no alternative antimalarial medicine is available yet with the same level of efficacy and tolerability as ACTs.

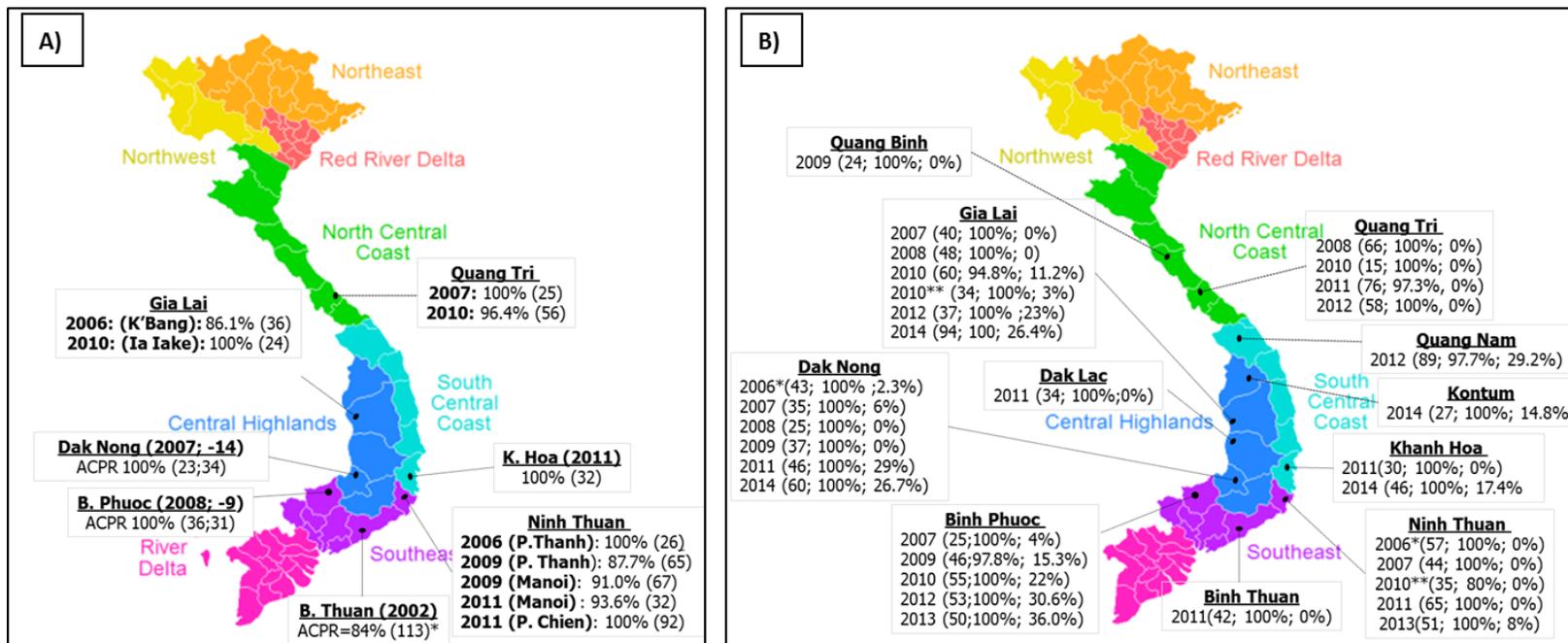


Figure 6. Evolution of antimalarial drug efficacy in Vietnam (2006 – 2015) [104, 105].

A) Retrospective data (2006-2014) on CQ efficacy for the treatment of uncomplicated *P. vivax* malaria in Central Vietnam (28-day ACPR; Source: NIMPE, Hanoi). B) Retrospective data (2006-2014) on ACTs (Dihydro-artemisinin 40mg + piperazine 320mg) efficacy for the treatment of uncomplicated *P. falciparum* malaria in Vietnam (42-day ACPR).

Numbers in brackets represent respectively, (number of patients studied; %ACPR; Day 3 positivity rate).

*Dihydroartemisinin 40mg + piperazine 320mg; **Artesunate + Azythromycin.

5. Malaria diagnosis methods and tools

Malaria is potentially a life threatening disease if the diagnosis and treatment is delayed, and this represents the main causes of malaria deaths in Sub-Saharan African countries [10, 11]. Early diagnosis and prompt treatment with effective antimalarial drugs is one of the key WHO recommended strategies for malaria control [10, 114]. Several diagnosis methods, from standard microscopy to advanced molecular methods are available today to confirm malaria infection.

5. 1. Clinical diagnosis

Clinical diagnosis is based on the patients' signs and symptoms gathered during the physical examination. Malaria symptoms are non-specific and variable, including fever and other flu-like symptoms such as headache, weakness, myalgia, chills, dizziness, abdominal pain, diarrhea, nausea, vomiting, etc. Therefore, the clinical diagnosis of malaria often leads to over-treatment with anti-malaria drugs [115]. Another major pitfall associated with clinical diagnosis is the occurrence of co-infections (*e.g.*, *P. falciparum* and *P. vivax*) and treatment for one may not treat the other. Therefore, clinical findings should always be confirmed by a laboratory test for malaria [116].

5.2. Light microscopy (LM)

Conventional light microscopy is considered as a “gold standard” for malaria parasite identification, allowing the identification of parasite species and stage, as well as quantification of parasite density [117]. Therefore, LM can be used not only for malaria

diagnosis confirmation but also to monitor antimalarial drug efficacy. The thick and/or thin blood smears are taken most often from a finger prick and are stained with a 5% Giemsa solution. The thick smears are most useful for screening the presence of parasites while thin blood smears identify malaria species [118]. Parasite density is determined by counting the number of asexual parasites per 200 white blood cells with a hand tally counter, and computed assuming 8000 WBCs per microliter of blood [119].

A trained and skilled microscopist can detect densities of 5-20 parasites/ μ l blood in approximately 60 minutes [120]. Though LM is considered a simple and inexpensive technique, the quality of the results varies depending on the skills of the microscopist, the quality of the slides, and the maintenance of the microscope, which if not adequate may lead to false positive or negative results. In addition, LM most of the time requires an electricity supply which is not available in all remote areas. An additional challenge that microscopists face is the accurate detection of mixed- and low-density infections.

In many malaria-endemic and low-income countries, LM is often not reliable due to the lack of training and supervision of microscopists, and the difficulties in retaining well-trained staff who are often underpaid and overworked in many health care programmes [121]. Conversely, in low- or non-endemic areas, in addition to low density infections, laboratory technicians are often unfamiliar with malaria and may miss the parasites.

5.3. Malaria rapid diagnostic tests (RDTs)

In order to overcome the above-mentioned limitations of LM, RDTs were developed and their performance was systematically tested by the WHO in collaboration with the Foundation for Innovative New Diagnostics (FIND) [122].

RDTs are based on an immune-chromatographic assay with monoclonal antibodies directed against the target parasite antigen and impregnated on a test membrane, allowing for the detection of malaria antigen in a small amount of blood. Malaria RDTs started to be developed in the mid-1990s [123] and are currently recommended by the WHO for parasitological confirmation before ACT treatment. The most commonly used RDTs to detect *P. falciparum* infections are based on the detection of histidine-rich protein 2 (HRP2), which is specifically expressed by this species. Other RDTs are based on the detection of the genus specific aldolase enzyme and the HRP2 to distinguish between *P. falciparum* and non-*P. falciparum* infections, e.g. NOW[®]ICT (Binax, USA; ICT Malaria Pf/Pv, AMRAD ICT, Australia). RDT based on parasite lactate dehydrogenase (pLDH) can distinguish between *P. falciparum* and *P. vivax* infections. Though some RDTs have been developed to detect *P. knowlesi* [124], most of the available RDTs do not specifically differentiate between the three less common malaria parasites species, i.e. *P. ovale*, *P. malariae* and *P. knowlesi* [125]. The sensitivity of RDTs to detect parasite densities of 200 parasites/ μ l varies between RDT brands and *Plasmodium* species. For example, RDTs against Pf HRP2 have a 95% sensitivity while for non-*P. falciparum* this is commonly around 85% [122, 125].

Unlike LM, RDTs results are obtained fast (in 15-20min), are easy to perform, and the reading requires only limited training. In addition, they do not require equipment investment or electricity. Therefore, they are highly suitable for malaria diagnosis at the community level, especially in poor and remote areas with limited access to good quality microscopy services. On the other hand, RDTs have the disadvantage of providing only qualitative results (no information on parasite density), while their accuracy can be affected by non-optimal storage conditions (high temperature and humidity) in the field.

An online tool has been developed (<http://www.rdt-interactive-guide.org/>) for NMCPs to choose the best RDT for their settings based on the results of the assessment of the WHO malaria RDT Product testing programme [125]. As a recommendation, the selected RDTs should have a panel detection score (PDS) of at least 75% at 200 parasites/ μ l for *P. falciparum* or *P. vivax*, and the false positive rate and invalid rate should be less than 10% and 5%, respectively.

False positive results of RDTs may be due to the persistence of circulating malaria antigens (HRP2, pLDH) in the circulation for 2-4 weeks after successful antimalaria treatment, presence of sexual but not asexual forms, and possible cross-reaction with rheumatoid factor [126-129]. False negative results are mainly due to low parasite density under the limit of detection or extremely high parasite density [130] and importantly, by HRP2-deficient *P. falciparum* strains, which have now been reported from different world regions including Southeast Asia [131-133]. Errors that may

impact RDT performance are related to test-line interpretation often due to unclear or even incorrect test instructions [134] or due to poor training of health workers. A recent call to harmonize labelling and instructions of malaria RDTs available in the market aims to improve user-friendliness and minimize errors due to different RDT performance [130].

Therefore, in research settings or for more detailed information on malaria infections, RDT results are complemented by LM to better characterize infections (species and density determination) and monitor treatment efficacy [121].

5.4. Molecular diagnosis of malaria

5.4.1. Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) based techniques have proven to be one of the most sensitive and specific methods for the detection of malaria infections, particularly those with mixed infections, low parasite density or drug resistant parasites [129, 135]. PCR techniques can detect as few as ≥ 0.002 parasite/ μL of blood compared to 50-100 parasites/ μL by LM or RDT [136-138]. Therefore, PCR-based techniques allow for an accurate quantification of the local parasite reservoir and a better understanding of its contribution to malaria transmission and dynamics [129, 139-141], and they are now frequently used as gold standard tools to evaluate other diagnostic tools [142].

PCR-based techniques can be designed to detect parasite DNA or RNA. DNA-based techniques include single-, nested-, multiplex-, and real-time PCR, [143, 144],

isothermal recombinase polymerase amplification (RPA)[145], and alternative PCR-based detection methods such as PET-PCR, PRET real-time PCR [146-148], etc. Other, PCR techniques such as quantitative reverse transcription PCR (qRT-PCR), nucleic acid sequence-based amplification (NASBA), and ELISA-like hybridization assays [136, 149, 150], are based on RNA detection in order to achieve higher sensitivity due to increased number of transcript target compared to DNA. However, the main limitation of these techniques is that RNA degrades faster than DNA and therefore requires special RNase inhibitor buffers for sample collection and storage, and special handling areas, and are thus difficult to implement in a field setting.

Most PCR-based techniques are designed to amplify multi-copy genes such as the small subunits 18S rRNA genes of the *Plasmodium* species, present in 5-8 copies per genome, which allows for species identification using species-specific oligonucleotide primers [151] Relevant to the present thesis, the semi-nested multiplex malaria PCR (SnM-PCR), which amplifies the 18S rRNA, is based in a primary PCR reaction that includes a universal reverse primer with two forward primers specific for Plasmodium and mammals and a nested PCR reaction that includes a *Plasmodium*-specific forward primer plus species-specific reverse primers for *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale* malaria parasites [143]. Other modified PCR methods such as nested PCR, real-time PCR, and reverse transcription PCR (RT-PCR) also allow for accurate species determination [152, 153] including *P. knowlesi* infections [2, 27, 154], and enable the quantification of parasite density.

Additionally, PCR target genes include cytochrome *b* mitochondrial gene (30-100 copies/parasite genome) [155], AMA1 [156], Pfmt869/Pgmt19 (20 copies/parasite genome) [157], Stevor multigene family (30 – 40 copies/parasite genome) [158, 159], and the var gene's acidic terminal sequence (Var ATS) (59 copies/parasite genome), which was recently described as target sequence in an ultra-sensitive qPCR proved to be ten times more sensitive than the standard PCR assay [151]. Table 2 summarizes the currently available PCR-based techniques with main characteristics.

Two main sampling strategies can be used for PCR analysis: (i) whole blood (directly processed or stored at -20°C), or (ii) whole blood spotted onto standard Whatman grade 3 filter paper or on Whatman Flinders Technology Associates (FTA) classic cards [54, 160]. To date, the most common sample collection method used in the field is dry blood spot filter paper (DBSF), which is collected from a finger prick onto various filter paper types [143, 161]. DBSF, particularly in large-scale epidemiological studies, has several advantages over venepuncture sample collection: it is not dependent on the availability of freezers (which may be limited in endemic areas), is less invasive and thus more acceptable at the community level, it is easy to perform and requires less training, and it is easy to store and transport [161, 162]. However, PCR sensitivity is also dependent on the parasite DNA concentration and therefore, the blood volume collected, thus PCR aiming to detect parasites on a 5-10 µl of whole blood, i.e. DBSF, will miss parasites compared to methods using 200 µl or 1 ml of whole blood [161-163]. As a result, in elimination contexts, where detection of every single *Plasmodium*

carrier is paramount, using sampling methods with larger blood volumes will increase PCR detection and capacity to accurately quantify low-density infections [160]. In low transmission settings, strategies such as pooling samples before PCR analysis have been used to reduce the costs in large epidemiological studies with limited resources [164].

However, the main drawback of PCR-based diagnostic techniques is the need for an advanced technological platform with complex equipment and reagents, well-trained personnel, and optimally functioning quality control and equipment maintenance systems, which are not available in all malaria-endemic areas. In addition, PCR costs, which include those of DNA extraction methods, are still unaffordable as routine diagnostic test in low-income settings where malaria is endemic [165]. Consequently, PCR diagnosis for malaria is mainly implemented in reference laboratories or for research purposes.

Table 2. Target gene and limitation of Plasmodium falciparum detection assay [151]

Method	Template Molecule	Target Gene	Quantification	Parasites/ μ l Blood
Nested PCR	DNA	18S rRNA, <i>dhfr-ts</i> , 28S rRNA, <i>stevor</i>	No	0.1–10
PCR	DNA	mitochondrial DNA	No	0.5
qPCR	DNA	18S rRNA, <i>cox1</i> , <i>cytb</i>	Yes	0.02–3
PCR-based	DNA	18S rRNA, <i>cox1</i>	Yes/No	0.5–1
LAMP ^a	DNA	18S rRNA, mitochondrial DNA	No	1–10
RPA ^a	DNA	18S rRNA	No	4
qRT-PCR	RNA	18S rRNA	Yes	0.002–0.02
(QT-)NASBA ^a	RNA	18S rRNA	Yes/No	0.02
Ultra-sensitive qPCR	RNA	TARE-2 and VarATS*	Yes	0.03-0.15

^aIsothermal amplification process. *TARE-2: Telomere associate repetitive element 2; VarATS: var gene's acidic terminal sequence.

5.4.2. Gametocyte detection

PCR-based techniques to detect RNA are also used to detect and quantify gametocytes to accurately quantify the human infectious reservoir. As the sensitivity of LM is limited to the detection of a very low-density gametocytaemia [166, 167], a variety of molecular methods such as RT-PCR, QT-NASBA, and reverse transcriptase loop mediated amplification (RT-LAMP) have been developed for gametocyte detection

[160, 168-170]. QT-NASBA and a qRT-PCR TaqMan probe-based assay, targeting the transcripts of the *P. falciparum* Pfs25 gene or its homologue in *Plasmodium vivax* (Pvs25), are the most commonly applied techniques for gametocyte detection of both *P. falciparum* and *P. vivax* [160, 167-169, 171]. In malaria elimination settings, understanding the prevalence and risk factors for gametocyte carriage can help to better target strategies for malaria elimination, as gametocyte carriers (even those with very low densities) can still maintain malaria transmission [172, 173]. Detection and treatment of all gametocyte carriage is necessary in order to reduce malaria transmission and prevent the selection and spread of resistant parasites [173]. However, as for other molecular methods, gametocyte detection is not easy to transfer to remote field laboratories.

5.4.3. Loop-mediated isothermal amplification (LAMP)

Loop-mediated isothermal amplification (LAMP) was first published in 2000 [174], as a simple and inexpensive amplification method to be implemented in basic field conditions (“field PCR”) for the detection of low-density parasite infections. The first development of LAMP assay detecting *P. falciparum* 18rRNA genes was done in 2006 [175], followed by a malaria-specific LAMP in 2007 [176] and a LAMP method targeting mitochondrial DNA in 2010 [157]. In 2013, a field-friendly LAMP kit was developed by FIND and a Japanese company (Eiken Chemical Co., Ltd) [177, 178]. The kits have been evaluated in laboratory and field conditions for both *P. falciparum* and *P. vivax* with

minimal training using either directly processed whole blood samples or blood collected on filter paper [178-180]. The LAMP reaction is shown in Figure 7.

Briefly, the main advantages of this technique are that *Bacillus stearotherophilus* DNA polymerase with strand displacement activity performs a one-step amplification, with a set of four specifically designed primers to identify six distinct sequences on the target DNA [174, 181]. The DNA amplification is performed at a fixed temperature through a repetition of two types of elongation reactions occurring at the loop regions, *i.e.* self-elongation of templates from the stem loop structure formed at the 3'-terminal, and the binding and elongation of new primers to the loop region (Figure 7). Moreover, LAMP amplification reaction time can be shortened by around one-third to one-half by using two extra primers (loop primers) [181]. LAMP amplifies a few to 10^9 copies in less than an hour and its sensitivity is comparable to PCR [182, 183]. In addition, it allows for easier visualization due to the release of pyrophosphate that causes turbidity (precipitation or by fluorescence) that creates a visible color change that can be seen by the naked eye. This also reduces the risk of DNA contamination, since reaction tubes post-amplification do not need to be opened.

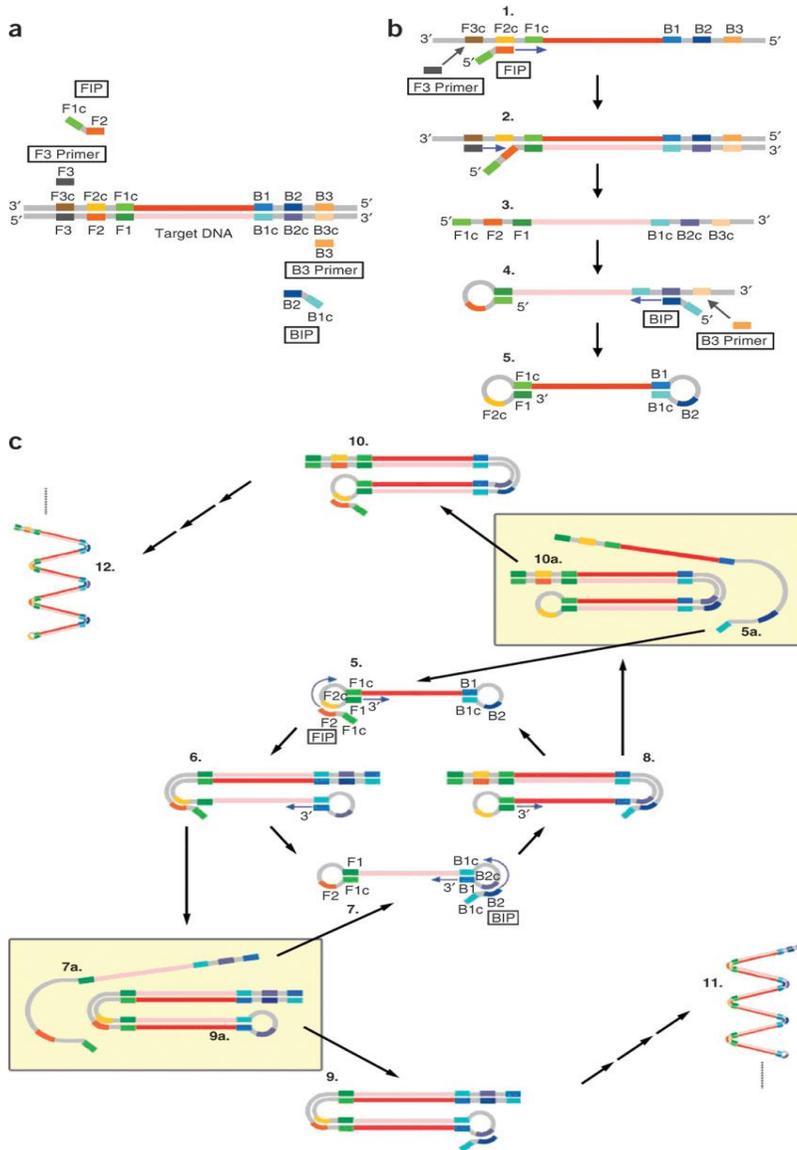


Figure 7. Basic amplification step of LAMP.

a) Primer design of the LAMP reaction (2 inner and 2 outer primers, plus 2 additional loop-primers), (b) Starting structure producing step (DNA synthesis starts from FIP and DNA amplification proceeds with BIP), (c) Cycling amplification step. Self-primed DNA synthesis is initiated from the 3' end F1 region, and the elongation starts from FIP annealing to the single strand of the F2c region in the loop structure. Passing through several steps, structures 5- 12 are generated [184].

Thus, LAMP characteristics, i.e. user-friendly, low-tech but high sensitivity, make it a promising diagnosis tool for field settings and elimination campaigns compared to PCR [175, 182, 185].

Despite all its advantages, LAMP techniques are not widely implemented in malaria endemic areas, as the technique remains costly (about 5.3 USD/reaction) [186] and with some degree of complexity that require basic laboratory skills (as compared to the simplicity of RDTs). In addition, contamination problems have been described in some reports [183, 187-190]. Even though the results are promising, optimization will be required to achieve the needs of a point of care (POC) diagnosis tool.

5.4.4. Malaria genotyping methods

In the context of malaria elimination, understanding genetic diversity and population structure of parasite populations is essential to design meaningful strategies and monitor the impact of those that have been implemented.

In recent years, genotyping techniques based on size polymorphic markers have become the method of choice for many molecular epidemiological studies [191, 192], aiming to unveil genetic diversity and population structure of human *Plasmodium* parasites worldwide [193, 194], mapping inherited traits in the progeny of a genetic cross [195], or improving the capacity to distinguish recrudescence/relapses from new infections [196]. The choice of markers depends on the aim of studies.

- *Antigen loci*

Highly polymorphic surface antigens, such as Merozoite Surface Proteins (MSP1 to 5) [197-199] and the circumsporozoite protein (CSP) [199, 200], (that contain both single nucleotide polymorphisms (SNPs) and tandem repeat copy number variations), have been widely used to differentiate individual clones within single infections [201] and measure parameters such as multiplicity of infection (MOI), and molecular Force of Infection (FOI), i.e. the number of new clones acquired over time [202], and thus describe the epidemiology of malaria parasites and impact of interventions. Polymorphic gene markers are less useful for interpreting population structure and genetic diversity, since these are subject to selection and therefore can converge at the population level [203].

- *Microsatellite genotyping*

Microsatellites (MS) are non-antigenic short tandem repeats in DNA sequences, typically including one to six nucleotides, with a variable number of repeats. MS are interspersed throughout the genome and originate at mutation events such as replication slippage and/or slip-strand mismatch repair, resulting in sequence length variation [204]. Variation in the repeat length causes size polymorphisms within the locus, which can be used to differentiate organisms in population diversity studies. Historically, MS have been extremely useful for investigating population genetics of several organisms such as *Toxoplasma*, *Leishmania*, *Trypanosoma* [205, 206], and importantly, human *Plasmodium spp* (*P. falciparum*, *P. vivax* and *P. malaria*) [207-210].

MS are highly abundant in the *P. falciparum* genome, which has an AT base-pair content of approximately 82% versus 60% in *P. vivax*, with an average of one MS locus every 2-3 kb [211-214]. Since 1999, when the first 12 MS for *P. falciparum* were described [215], MS genotyping has been extensively used to detect naturally-occurring multi-clone infections, analyze the genetic diversity, population structure, and transmission dynamics of parasite populations [193, 207, 216-218], trace the geographic origin infections, measure the impact of interventions, and to investigate the origin and spread of drug resistant parasites and its molecular markers [219, 220].

The use of MS in *P. vivax* parasites followed that of *P. falciparum*. Over the past decade, at least 240 MS have been identified in the *P. vivax* genome, usually by *in silico* methods [194]. Among all known *P. vivax* MS markers, 42 of them have been tested in more than one field site [194, 208, 209], and their quality was recently validated based on population parameters (*i.e.*, endemicity, sample size), MS parameters (*i.e.*, motif length, repeat length, repeat type and genomic position), and genetic diversity indices (*ie*, number of alleles per locus and expected heterozygosity (*He*) and repeat length size) [139]. A total of 18 MS were identified as ideal candidates for measuring population diversity between global regions but only nine MS with balanced diversity (PvMS7, 3.502, MS4, MS6, MS9, MS10, MS12, MS20, 14.279) were highly recommended for measuring population diversity, as it can avoid bias from excess or reduced diversity. Five additional markers (3.27, 8.504, PvMS11, MS16, MS8) were identified as candidates for MOI studies as those MS markers have significant excess

diversity in more than one test category, and two MS (3.27, MS8) more frequently used were considered perfect for calculating multiplicity of infections (MOI) in *P. vivax* populations [194] (Figure 8).

As for *P. falciparum*, *P. vivax* MS have been used in a wide range of epidemiological studies, from those investigating transmission dynamics in longitudinal cohorts [191, 194, 221, 222], genetic diversity [223-225], and population structure [222, 226, 227], to the contribution of relapses to the burden of infection in special study designs [228, 229]. However, in contrast to *P. falciparum*, MS genotyping in *P. vivax* parasites cannot be used to differentiate between recurrent and new infections in drug efficacy studies since both can harbor new genotypes compared with the primary infection [230] .

Summary of results for individual analyses								
MS Marker	Endemicity vs. Diversity	Motif Lengths. Diversity	Repeat Lengths. Diversity	Repeat Typevs. Diversity	Genomic Positionvs. Diversity	Recommendation	Chr. (priority)	Ref.
PvMS7	Balanced	Balanced	Balanced	Balanced	Balanced	1° Panel	2 (A)	[31]
3.502	Balanced	Balanced	Balanced	Balanced	Balanced	1° Panel	3 (A)	[28]
3.503	Balanced	Balanced	Balanced	Balanced	Balanced	1° Panel	3 (B)	[28]
MS1	Balanced	Balanced	Balanced	Balanced	Balanced	1° Panel	3 (C)	[30]
ms033 (PvMS5)	Balanced	Balanced	Balanced	Balanced	Balanced	1° Panel	3 (D)	[29, 31]
MS12	Balanced	Balanced	Balanced	Balanced	Balanced	1° Panel	5 (A)	[30]
MS15	Balanced	Balanced	Balanced	Balanced	Balanced	1° Panel	5 (B)	[30]
MS4 (ms050)	Balanced	Balanced	Balanced	Balanced	Balanced	1° Panel	6 (A)	[29, 30]
ms038 (PvMS9)	Balanced	Balanced	Balanced	Balanced	Balanced	1° Panel	6 (B)	[29, 31]
MS9 (Pv6635)	Balanced	Balanced	Balanced	Balanced	Balanced	1° Panel	8 (A)	[29, 32]
MS20 (ms116)	Balanced	Balanced	Balanced	Balanced	Balanced	1° Panel	10 (A)	[29, 30]
MS6	Balanced	Balanced	Balanced	Balanced	Balanced	1° Panel	11 (A)	[30]
11.162	Balanced	Balanced	Balanced	Balanced	Balanced	1° Panel	11 (B)	[28]
MS10	Balanced	Balanced	Balanced	Balanced	Balanced	1° Panel	13 (A)	[30]
13.239	Balanced	Balanced	Balanced	Balanced	Balanced	1° Panel	13 (B)	[28]
PvMS8	Balanced	Balanced	Balanced	Balanced	Balanced	1° Panel	13 (C)	[31]
14.297	Balanced	Balanced	Balanced	Balanced	Balanced	1° Panel	14 (A)	[28]
PVMS6	Balanced	Balanced	Balanced	Balanced	Balanced	1° Panel	14 (B)	[31]
PvMS2	Balanced	Reduced (p < 0.03)	Balanced	Balanced	Balanced	2° Panel	3 (A)	[31]
MS2	Balanced	Balanced	Balanced	Balanced	Excess (p < 0.02)	2° Panel	6 (A)	[30]
MS5	Balanced	Balanced	Reduced (p < 0.05)	Balanced	Balanced	2° Panel	6 (B)	[30]
6.34	Balanced	Balanced	Balanced	Balanced	Reduced (p < 0.05)	2° Panel	6 (C)	[28]
PvMS4	Balanced	Excess (p < 0.003)	Balanced	Balanced	Balanced	2° Panel	6 (D)	[31]
ms196 (PvMS3)	Balanced	Excess (p < 0.04)	Balanced	Balanced	Balanced	2° Panel	8 (A)	[29, 31]
Pvsal1814	Balanced	Excess (p < 0.02)	Balanced	Balanced	Balanced	2° Panel	14 (A)	[32]
3.27	Excess (p < 0.02)	Excess (p < 0.005)	Excess (p < 0.004)	Excess (p < 0.02)	Excess (p < 0.05)	MOI	3 (A)	[28]
8.504	Excess (p < 0.03)	Balanced	Balanced	Excess (p < 0.03)	Excess (p < 0.01)	MOI	8 (A)	[28]
PvMS11	Excess (p < 0.03)	Balanced	Excess (p < 0.006)	Excess (p < 0.01)	Balanced	MOI	8 (B)	[31]
MS16	Excess (p < 0.0001)	Excess (p < 0.0004)	Excess (p < 0.05)	Balanced	Excess (p < 0.001)	MOI	9 (A)	[30]
MS8 (ms206)	Excess (p < 0.03)	Excess (p < 0.05)	Excess (p < 0.03)	Balanced	Balanced	MOI	12 (A)	[29, 30]
1.501	Balanced	Reduced (p < 0.02)	Balanced	Balanced	Reduced (p < 0.02)	Exclude	1 (A)	[28]
MS3	Reduced (p < 0.05)	Balanced	Balanced	Balanced	Reduced (p < 0.02)	Exclude	4 (A)	[30]
PvMS10	Balanced	Reduced p < 0.003	Reduced p < 0.01	Reduced p < 0.01	Balanced	Exclude	5 (A)	[31]
MS7	Reduced (p < 0.01)	Reduced (p < 0.006)	Reduced (p < 0.05)	Reduced (p < 0.02)	Reduced (p < 0.02)	Exclude	12 (A)	[30]
PvMS1	Balanced	Reduced (p < 0.04)	Balanced	Balanced	Reduced (p < 0.02)	Exclude	12 (B)	[31]

Figure 8. Summary of statistically validated *P. vivax* MS for usage in population diversity and MOI studies.

“1° Panel” indicates balanced diversity in all six test categories and usage as the primary panel of markers for measuring population diversity. “2° Panel” indicates markers with significant excess or reduction of diversity in one of six test categories. “Exclude” indicates markers with significant reduction in diversity in more than one of the six test categories. These markers are not recommended, as they consistently result in a misrepresentation of population diversity

due to reduced polymorphic potential. "MOI" (multiplicity of infection) indicates MS markers that consistently have significant excess diversity in more than one test category. For chromosomes with more than one MS marker tested, priority has been assigned (A-D). Priority is based on the total number of studies that have utilized the marker, with a higher priority being placed on markers that have been used more frequently. Bold font indicates markers of highest priority [194].

Even though more affordable than other methodologies such as WGS, the costs associated with MS genotyping (which includes fluorophore-labelled primers, the use of a capillary electrophoresis platform typically a Sanger-type sequencing machine) are still high. In addition, variability in the MS used for analysis together with different parameters and protocols used for the analysis (i.e, different rules and cut-offs to identify true peaks, different software to access genetic diversity values, different number of markers used, and the occurrence of missing data [192, 194, 225, 231-234] can lead to substantial variation between studies and limit the comparability of the obtained results [235].

- *Barcode / Single nucleotide polymorphism genotyping (SNPs)*

An SNP is a single-base pair mutation at a specific locus, usually consisting of two alleles (where the rare allele frequency is >1%). Genome sequencing has identified more than 112,000 SNPs from about 18 parasite genomes of the *P. falciparum* parasite [236, 237]. Current technology has made genotyping of SNPs by real-time PCR (using dual probes in an end-point detection assay) a standard practice [236].

An SNP “barcode” contains a combination of SNPs that together express the unique pattern of variation for the parasite sequence [238]. SNP barcoding is known as a highly sensitive technique (with a 99% success rate) in distinguishing different parasite genotypes, while requiring a very small amount of DNA in infected blood samples [238]. SNP barcoding is useful for malaria elimination programmes as the assay can be used to differentiate parasites from different geographical areas and imported cases, to monitor the frequency and distribution of specific parasites in the population, to distinguish recrudescence from re-infection in drug trials, as well as for monitoring the adaption of samples in the laboratory, or assessing changes that a population may be undergoing due to external pressures [239]. The barcode panel should include SNPs representative of the genetic diversity present in the parasite population from the geographical area where the panel is to be used, and should be re-evaluated if this is to be used in a different malaria-endemic region.

The advantage of SNP genotyping is that it can be carried out using several different platforms that vary in the chemistries used [240]. For example, high-resolution melting (HRM) SNP barcoding is a simple, rapid and low-cost SNP genotyping method based on amplicon melting [241], which requires only a saturating double stranded DNA dye and a post-PCR short melting step, limited training, and has minimal subjective data due to its visualization software [238]. Compared to other molecular methods such as MS genotyping, barcode genotyping can easily detect possible cross-contaminations, thus improving laboratory quality standards. The technique can also be standardized

and calibrated across multiple studies, in order to obtain faster sample analysis, less subjectivity [194, 231, 236], and to allow for global comparisons and exploration of patterns over a long time due to the method's high reproducibility [242].

However, SNPs reflect common alleles rather than rare ones, when analysis involves a small number of samples [243], and have lower sensitivity than MS in detecting recent events due to their lower mutation rate [244]. In addition, SNP barcoding is also challenging in areas with a high proportion of multi-clonal infections [245] and ascertainment of issues with bias problem [243, 246]. As MS provides a higher resolution than SNP barcoding, due to a higher polymorphic potential (and lower cost of reagents [194, 231, 236]), a combination of MS markers and SNP barcoding could potentially become a useful population genomics approach to identify loci linked to artemisinin resistance or to multiple origins of drug-resistant haplotypes in areas of low transmission [247].

- Genomics

WGS has greatly advanced our understanding of the global genetic diversity of *Plasmodium* parasites, and is an important tool to identify markers of drug resistance [194, 240]. In low transmission areas, where genetic diversity, especially of *P. falciparum* parasite populations, is decreasing, WGS is also a potential tool to differentiate recrudescence infections in clinical trials.

WGS of *Plasmodium spp* is challenged by the extremely AT-rich regions of the *P. falciparum* genome (80.6% AT) [240], the need for a good quantity and quality of

starting material (which is difficult in clinical samples collected on filter paper and contaminating human DNA) and the added difficulty in analysing polyclonal and mixed- infections samples. Several protocols have been developed to overcome those challenges: most current protocols include CF11 column filtration to remove human leukocytes (the source of contaminating human DNA), or 48-hour *ex vivo* schizont maturation assays to increase the amount of Plasmodium DNA [248, 249], amplification and enrichment protocols [250, 251] .

Indeed, WGS of *P. falciparum* laboratory-adapted strains under drug pressure have been used to identify K13 as artemisinin resistant marker [67], while WGS of *P. vivax* parasites has been very useful to construct global genetic diversity maps, and better understand parasite biology [240] while *P. vivax* continuous culture is still lacking.

As prices for WGS techniques continue to decrease, WGS will become more widely used and will allow comparison of the genetic diversity of worldwide populations with a higher resolution than by MS or SNPs [252]. However, these techniques are not easily deployed as they require extremely expensive equipment and reagents, and because the challenges represented by training, technology transfer, complexity of study design, data analysis, and interpretation make genomics technology unsuitable in low-income settings or in field-based conditions.

5.5. Serological diagnosis

Serological assays for malaria are based on the detection of antibodies against asexual or sexual blood stages of malaria parasites, using either an indirect immunofluorescence antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA), or multiplex assays.

Plasmodium spp infections trigger the synthesis of specific antibodies (especially of the IgM and IgG isotypes) within 1-2 weeks after initial infection, which progressively decline following the primary infection [253]. The antibody response to Plasmodium is cumulative and long lasting, developing after repeated exposures to the parasite and persisting for months or years after infection has resolved [254]. It also varies according to age in a given area, and for the same age group it varies between areas of different transmission intensity.

Due to the time required for development, as well as the persistence of antibodies, malaria serology does not detect acute infection but rather measures past exposure to malaria parasites, hence it has been proven useful for measuring malaria transmission intensity, especially at low transmission levels where other techniques (e.g., LM, entomological inoculation rate, PCR) become less sensitive and costly. It is also used in epidemiological surveys, for screening potential blood donors, or to provide evidence of recent infection in non-immune travellers [255-258]. The main disadvantage of serology has long been the lack of standardization in the antigens and

methodologies used, but this has been recently improved by the advent of recombinant proteins and high-throughput screening methods [259].

IFAT was considered until recently as the gold standard for malarial serology testing [253, 260] and has long been used as a tool for detecting Plasmodium-specific antibodies in blood banks [261]. IFAT uses pre-coated slides with either specific or crude antigens (stored at -30°C until used) and quantifies both IgG and IgM malaria antibodies in patients' sera. This is done by detecting antigen-antibody (Ag-Ab) complexes marked with fluorescein-labelled anti-human antibodies, and requires a fluorescence microscope and well-trained technicians. Despite its simplicity and sensitivity, the technique remains impractical for routine use due to its lack of automation and reproducibility. Therefore, ELISA methods using recombinant antigens fill the gap by being easily standardized and automated, allowing for the testing of large numbers of serum samples (using 96-well coated plates) in a short time and at low cost, together with higher sensitivity and specificity levels [262]. However, this technique can only be used for a limited number of antigens.

Multiplex assays are preferred for high-throughput screening of malaria immunity because they are cost- and time-effective and minimize the sample volume requirements [263]. Currently, multiplex bead assays, such as Luminex technology have been developed by Luminex® Corporation and allows the simultaneous testing of several serological markers [264-266]. Luminex are described to have similar or improved sensitivity relative to ELISA assays [267], but have better reproducibility and

a greater dynamic range. Recently, high-throughput proteomic microarray technology has been applied in order to identify the targets of broad antibody responses to *P. falciparum* and identify sensitive serological markers in different malaria transmission intensities [268].

In the context of malaria elimination, serology becomes essential for measuring individual variations in antibody responses, the occurrence of multiple malaria parasite antigens, as well as for increasing the probability of measuring changes in antibody responses by combining different markers [269]. This approach has been shown to be useful for evaluating the impact of interventions, identifying hotspots of transmission and sub-populations in high-risk infections [270, 271], estimating transmission levels and related historical changes, confirming malaria elimination, and monitoring re-emergence of malaria [259]. Despite all its advantages, a number of knowledge gaps need to be addressed before obtaining an optimized sero-surveillance tool, i.e. choice of the type and number of antigens for each Plasmodium parasite in different transmission conditions, sensitivity and specificity of multiplex serological assays, factors affecting the development and maintenance of antibody and the longevity of antibody responses [259]. However, serology still offers a simple and sensitive tool that could be combined with other tools in malaria surveillance and geospatial analysis to achieve malaria elimination.

At the moment of starting, the laboratory work included in this PhD thesis (2008), malaria in Vietnam was still in the control phase and reporting of parasitological data

was heavily based on LM [272-275]. National monitoring of antimalarial drug efficacy was done at sentinel sites, however no *P. falciparum* resistance to artemisin and *P. vivax* resistance to CQ had been reported [104]. In the context of the PhD and with the support of the bilateral collaboration with ITM, NIMPE implemented molecular methods such as ELISAs, PCR-based diagnostic methods (SS-PCR, modified semi-Nested PCR and qPCR) and MS genotyping protocols to accurately investigate epidemiology of human malaria species; assess genetic diversity and population structure of *P. vivax* populations and evaluate parasite clearance and prevalence of K13 mutations in Vietnam. This contribution played an important role in the success of NMCP to move from control phase to pre-malaria-elimination phase.

References

1. Rowe JA, Claessens A, Corrigan RA, Arman M: Adhesion of *Plasmodium falciparum*-infected erythrocytes to human cells: molecular mechanisms and therapeutic implications. *Expert Rev Mol Med* 2009, 11:e16.
2. Cox-Singh J, Davis TM, Lee KS, Shamsul SS, Matusop A, Ratnam S, Rahman HA, Conway DJ, Singh B: *Plasmodium knowlesi* malaria in humans is widely distributed and potentially life threatening. *Clin Infect Dis* 2008, 46:165-171.
3. Cogswell FB: The hypnozoite and relapse in primate malaria. *Clin Microbiol Rev* 1992, 5:26-35.
4. Winzeler EA: Malaria research in the post-genomic era. *Nature* 2008, 455:751-756.
5. Collins WE, Jeffery GM: *Plasmodium ovale*: parasite and disease. *Clin Microbiol Rev* 2005, 18:570-581.
6. Collins WE, Jeffery GM: *Plasmodium malariae*: parasite and disease. *Clin Microbiol Rev* 2007, 20:579-592.
7. Wassmer SC, Taylor TE, Rathod PK, Mishra SK, Mohanty S, Arevalo-Herrera M, Duraisingh MT, Smith JD: Investigating the Pathogenesis of Severe Malaria: A Multidisciplinary and Cross-Geographical Approach. *Am J Trop Med Hyg* 2015, 93:42-56.
8. Rajahram GS, Barber BE, William T, Grigg MJ, Menon J, Yeo TW, Anstey NM: Falling *Plasmodium knowlesi* Malaria Death Rate among Adults despite Rising Incidence, Sabah, Malaysia, 2010-2014. *Emerg Infect Dis* 2016, 22:41-48.
9. Rahimi BA, Thakkinstian A, White NJ, Sirivichayakul C, Dondorp AM, Chokejindachai W: Severe vivax malaria: a systematic review and meta-analysis of clinical studies since 1900. *Malar J* 2014, 13:481.

10. World Health Organization (WHO): World Malaria Report 2015. Available from: <http://www.who.int/malaria/publications/world-malaria-report-2015/en/>. Last accessed 9th Dec, 2015. Geneva 2015.
11. World Health Organization (WHO): World malaria report 2014. Available from: http://www.who.int/malaria/publications/world_malaria_report_2014/report/en/. Last accessed 12th Dec, 2015 2014
12. WHO certifies Sri Lanka malaria-free. Available from: <http://www.searo.who.int/mediacentre/releases/2016/1631/en/>. Last accessed on 26th November, 2016
13. U.S. Agency for International Development: President's malaria initiative, Greater Mekong Subregion malaria operational Plan FY 2015. Available from: <http://aplma.org/upload/resource/Documents/fy-2015-greater-mekong-subregion-malaria-operational-plan.pdf>. Last accessed 9th Dec, 2015 2015.
14. Hii J, Rueda LM: Malaria vectors in the Greater Mekong Subregion: overview of malaria vectors and remaining challenges. *Southeast Asian J Trop Med Public Health* 2013, 44 Suppl 1:73-165; discussion 306-167.
15. Trung HD: Malaria vector in Southeast Asia: identification, malaria transmission, behaviour and control *University of Antwerp and Institute of Tropical Medicine, Antwerp Belgium* 2003.
16. Peeters Grietens K, Gryseels C, Dierickx S, Bannister-Tyrrell M, Trienekens S, Uk S, Phoeuk P, Suon S, Set S, Gerrets R, et al: Characterizing Types of Human Mobility to Inform Differential and Targeted Malaria Elimination Strategies in Northeast Cambodia. *Sci Rep* 2015, 5:16837.
17. Nguyen HV, van den Eede P, van Overmeir C, Thang ND, Hung le X, D'Alessandro U, Erhart A: Marked age-dependent prevalence of symptomatic and patent infections and complexity of distribution of human *Plasmodium* species in central Vietnam. *Am J Trop Med Hyg* 2012, 87:989-995.

18. Imwong M, Stepniewska K, Tripura R, Peto TJ, Lwin KM, Vihokhern B, Wongsan K, von Seidlein L, Dhorda M, Snounou G, et al: Numerical Distributions of Parasite Densities During Asymptomatic Malaria. *J Infect Dis* 2016, 213:1322-1329.
19. Baum E, Sattabongkot J, Sirichaisinthop J, Kiattibutr K, Jain A, Taghavian O, Lee MC, Huw Davies D, Cui L, Felgner PL, Yan G: Common asymptomatic and submicroscopic malaria infections in Western Thailand revealed in longitudinal molecular and serological studies: a challenge to malaria elimination. *Malar J* 2016, 15:333.
20. Price RN, von Seidlein L, Valecha N, Nosten F, Baird JK, White NJ: Global extent of chloroquine-resistant *Plasmodium vivax*: a systematic review and meta-analysis. *Lancet Infect Dis* 2014, 14:982-991.
21. Fairhurst RM, Dondorp AM: Artemisinin-Resistant *Plasmodium falciparum* Malaria. *Microbiol Spectr* 2016, 4.
22. Hung NM: Situation of Malaria control in Vietnam during five years 2006-2010 Hanoi, Vietnam Medical publisher. Number: 240-2011/CXB/28-20/YH; 2011. Internal report
23. National Malaria Control Programme (NMCP): Annual malaria report in 2015 and malaria plan in 2016. pp. 81: National Institute of Malariology Parasitology, Entomology, Hanoi, Vietnam. Internal report 2016:81.
24. The Prime Minister of the Socialist Republic of Vietnam: Approving the national strategy for malaria prevention, control and eradication in Vietnam in the 2011-2020 period, with orientations toward 2030. Available from: http://www.chinhphu.vn/portal/page/portal/chinhphu/congdan/DuThaoVanBan?piref135_27935_135_27927_27927.mode=detail&piref135_27935_135_27927_27927.id=538. Last accessed 7thDec DNQ-T ed. Hanoi: Ministry of Health 2011.

25. Tran Thanh Duong NDT: Malaria epidemiological stratification in Vietnam 2014. Hanoi, Vietnam: Medical publisher. ISBN: 978-604-66-0905-6; 2015. Internal report.
26. National Malaria Control Programme (NMCP): Annual Malaria Report 2014. Hanoi, Vietnam: National Institute of Malariology, Parasitology and Entomology (NIMPE); 2015. Internal report.
27. Van den Eede P, Van HN, Van Overmeir C, Vythilingam I, Duc TN, Hung le X, Manh HN, Anne J, D'Alessandro U, Erhart A: Human *Plasmodium knowlesi* infections in young children in central Vietnam. *Malar J* 2009, 8:249.
28. Garros C, Van Nguyen C, Trung HD, Van Bortel W, Coosemans M, Manguin S: Distribution of *Anopheles* in Vietnam, with particular attention to malaria vectors of the *Anopheles minimus* complex. *Malar J* 2008, 7:11.
29. Ministry of Health, National Institute of Malariology Parasitology, Entomology (NIMPE), Hanoi, Vietnam: Guiderlines for malaria diagnosis and treatment *Ministry of Health, Vietnam 2016, number 4845/QD- BYT date 8/9/2016 (Vietnamese version)* 2016.
30. World Health Organization (WHO): World Malaria Report 2009. Available from: http://apps.who.int/iris/bitstream/10665/44234/1/9789241563901_eng.pdf. Last accessed 11thNov, 2016. Geneve, Swenden 2009.
31. The malERA Consultative Group on Vector control: A research agenda for malaria eradication: vector control. *PLoS Med* 2011, 8:e1000401.
32. Alonso PL, Brown G, Arevalo-Herrera M, Binka F, Chitnis C, Collins F, Doumbo OK, Greenwood B, Hall BF, Levine MM, et al: A research agenda to underpin malaria eradication. *PLoS Med* 2011, 8:e1000406.
33. World Health Organization (WHO): Malaria control to Malaria elimination: A manual for Elimination scenario planing. Available from:

- http://apps.who.int/iris/bitstream/10665/112485/1/9789241507028_eng.pdf.
Last accessed 7thDec, 2015. 2014.
34. 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.
 35. World Health Organization (WHO): Global malaria control and elimination: report of a technical review. Available from: http://apps.who.int/iris/bitstream/10665/43903/1/9789241596756_eng.pdf.
Last accessed 12th Dec, 2015. Geneva2008.
 36. Mendis K, Rietveld A, Warsame M, Bosman A, Greenwood B, Wernsdorfer WH: From malaria control to eradication: The WHO perspective. *Trop Med Int Health* 2009, 14:802-809.
 37. World Health Organization (WHO): Roll Back Malaria Partnership: Progress and impact series. Elimating malaria: learning from the past looking ahead. Available from: http://www.rollbackmalaria.org/microsites/ProgressImpactSeries/docs/report_9-en.pdf. Last access 11 July,2016. 2011.
 38. Trigg PI, Kondrachine AV: Commentary: malaria control in the 1990s. *Bull World Health Organ* 1998, 76:11-16.
 39. Global malaria control strategy. *Bull Pan Am Health Organ* 1993, 27:280-283.
 40. World Health Organization (WHO): Roll Back Malaria Partnership. The global malaria action plan. Available from <http://www.rbm.who.int/gmap/gmap.pdf> .
Last access 17 June 2010. 2008.
 41. Alonso PL, Tanner M: Public health challenges and prospects for malaria control and elimination. *Nat Med* 2013, 19:150-155.
 42. World Health Organization (WHO): Global technique strategy for malaria 2016-2030. Available from:

http://apps.who.int/iris/bitstream/10665/176712/1/9789241564991_eng.pdf.

Last access 11 July 2016. 2015.

43. Cui L, Yan G, Sattabongkot J, Cao Y, Chen B, Chen X, Fan Q, Fang Q, Jongwutiwes S, Parker D, et al: Malaria in the Greater Mekong Subregion: heterogeneity and complexity. *Acta Trop* 2012, 121:227-239.
44. Xu X, Zhou G, Wang Y, Hu Y, Ruan Y, Fan Q, Yang Z, Yan G, Cui L: Microgeographic Heterogeneity of Border Malaria During Elimination Phase, Yunnan Province, China, 2011-2013. *Emerg Infect Dis* 2016, 22:1363-1370.
45. Cui L, Yan G, Sattabongkot J, Chen B, Cao Y, Fan Q, Parker D, Sirichaisinthop J, Su XZ, Yang H, et al: Challenges and prospects for malaria elimination in the Greater Mekong Subregion. *Acta Trop* 2012, 121:240-245.
46. World Health Organization (WHO): Strategy for malaria elimination in Greater Mekong Suregion (2015-2030). Available from: http://iris.wpro.who.int/bitstream/handle/10665.1/10945/9789290617181_eng.pdf?sequence=1. Last accessed 18thDec, 2015. 2015.
47. World Health Organization (WHO): Malaria control today. Available from: http://www.who.int/malaria/publications/atoz/mct_workingpaper/en/. Last accessed 12thDec, 2015. Geneva, Switzerland2005
48. Wit Vd: Roll Back Malaria Initiative in Greater Me Krong subregion. Available from: <http://www.adb.org/sites/default/files/project-document/68470/34189-reg-tacr.pdf>. Last accessed 7thDec, 2015. 2005.
49. Dondorp AM, Nosten F, Yi P, Das D, Phyto AP, Tarning J, Lwin KM, Arie F, Hanpithakpong W, Lee SJ, et al: Artemisinin resistance in *Plasmodium falciparum* malaria. *N Engl J Med* 2009, 361:455-467.
50. World Health Organization (WHO): Emergency response to artemisinin resistance in the Greater Mekong subregion. Regional Framework for Action 2013-2015. Available from:

- <http://www.who.int/malaria/publications/atoz/9789241505321/en/>. Last accessed 7thDec, 2015. 2013.
51. World Health Organization (WHO): Global plan for artemisinin resistance containment (GPARC). <http://www.who.int/malaria/publications/atoz/9789241500838/en/> 2011.
 52. World Health Organization (WHO): Status report on artemisinin and ACT resistance. Available from: <http://www.who.int/malaria/publications/atoz/status-rep-artemisinin-resistance-sep2014.pdf>. Last accessed 7thDec, 2015. 2015.
 53. Nguyen Van Chuong HDT: Mass Media launched to respondes Word Malaria Day in Vietnam. Available from: <http://www.impe-qn.org.vn/impe-qn/vn/portal/InfoDetail.jsp?area=58&cat=1179&ID=9774>. Last access 16 July 2016. 2016.
 54. Thanh PV, Hong NV, Van Van N, Van Malderen C, Obsomer V, Rosanas-Urgell A, Grietens KP, Xa NX, Bancone G, Chowwiwat N, et al: Epidemiology of forest malaria in Central Vietnam: the hidden parasite reservoir. *Malar J* 2015, 14:86.
 55. White N: Antimalarial drug resistance and combination chemotherapy. *Philos Trans R Soc Lond B Biol Sci* 1999, 354:739-749.
 56. Denis MB, Tsuyuoka R, Poravuth Y, Narann TS, Seila S, Lim C, Incardona S, Lim P, Sem R, Socheat D, et al: Surveillance of the efficacy of artesunate and mefloquine combination for the treatment of uncomplicated falciparum malaria in Cambodia. *Trop Med Int Health* 2006, 11:1360-1366.
 57. Alker AP, Lim P, Sem R, Shah NK, Yi P, Bouth DM, Tsuyuoka R, Maguire JD, Fandeur T, Arieu F, et al: Pfm^{dr1} and in vivo resistance to artesunate-mefloquine in falciparum malaria on the Cambodian-Thai border. *Am J Trop Med Hyg* 2007, 76:641-647.

58. Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM, Artemisinin Resistance in Cambodia 1 Study C: Evidence of artemisinin-resistant malaria in western Cambodia. *N Engl J Med* 2008, 359:2619-2620.
59. Ashley EA, Dhorda M, Fairhurst RM, Amaratunga C, Lim P, Suon S, Sreng S, Anderson JM, Mao S, Sam B, et al: Spread of artemisinin resistance in *Plasmodium falciparum* malaria. *N Engl J Med* 2014, 371:411-423.
60. Phyto AP, Nkhoma S, Stepniewska K, Ashley EA, Nair S, McGready R, Ier Moo C, Al-Saai S, Dondorp AM, Lwin KM, et al: Emergence of artemisinin-resistant malaria on the western border of Thailand: a longitudinal study. *Lancet* 2012, 379:1960-1966.
61. Kyaw MP, Nyunt MH, Chit K, Aye MM, Aye KH, Aye MM, Lindegardh N, Tarning J, Imwong M, Jacob CG, et al: Reduced susceptibility of *Plasmodium falciparum* to artesunate in southern Myanmar. *PLoS One* 2013, 8:e57689.
62. Hien TT, Thuy-Nhien NT, Phu NH, Boni MF, Thanh NV, Nha-Ca NT, Thai le H, Thai CQ, Toi PV, Thuan PD, et al: In vivo susceptibility of *Plasmodium falciparum* to artesunate in Binh Phuoc Province, Vietnam. *Malar J* 2012, 11:355.
63. Dondorp AM, Yeung S, White L, Nguon C, Day NP, Socheat D, von Seidlein L: Artemisinin resistance: current status and scenarios for containment. *Nat Rev Microbiol* 2010, 8:272-280.
64. Carrara VI, Zwang J, Ashley EA, Price RN, Stepniewska K, Barends M, Brockman A, Anderson T, McGready R, Phaiphun L, et al: Changes in the treatment responses to artesunate-mefloquine on the northwestern border of Thailand during 13 years of continuous deployment. *PLoS One* 2009, 4:e4551.
65. Witkowski B, Lelievre J, Barragan MJ, Laurent V, Su XZ, Berry A, Benoit-Vical F: Increased tolerance to artemisinin in *Plasmodium falciparum* is mediated by a quiescence mechanism. *Antimicrob Agents Chemother* 2010, 54:1872-1877.

66. Witkowski B, Amaratunga C, Khim N, Sreng S, Chim P, Kim S, Lim P, Mao S, Sopha C, Sam B, et al: Novel phenotypic assays for the detection of artemisinin-resistant *Plasmodium falciparum* malaria in Cambodia: in-vitro and ex-vivo drug-response studies. *Lancet Infect Dis* 2013, 13:1043-1049.
67. Arieu F, Witkowski B, Amaratunga C, Beghain J, Langlois AC, Khim N, Kim S, Duru V, Bouchier C, Ma L, et al: A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. *Nature* 2014, 505:50-55.
68. Witkowski B, Khim N, Chim P, Kim S, Ke S, Kloeung N, Chy S, Duong S, Leang R, Ringwald P, et al: Reduced artemisinin susceptibility of *Plasmodium falciparum* ring stages in western Cambodia. *Antimicrob Agents Chemother* 2013, 57:914-923.
69. Straimer J, Gnadig NF, Witkowski B, Amaratunga C, Duru V, Ramadani AP, Dacheux M, Khim N, Zhang L, Lam S, et al: Drug resistance. K13-propeller mutations confer artemisinin resistance in *Plasmodium falciparum* clinical isolates. *Science* 2015, 347:428-431.
70. Menard D, Khim N, Beghain J, Adegnikaa AA, Shafiul-Alam M, Amodu O, Rahim-Awab G, Barnadas C, Berry A, Boum Y, et al: A Worldwide Map of *Plasmodium falciparum* K13-Propeller Polymorphisms. *N Engl J Med* 2016, 374:2453-2464.
71. Amaratunga C, Sreng S, Suon S, Phelps ES, Stepniewska K, Lim P, Zhou C, Mao S, Anderson JM, Lindegardh N, et al: Artemisinin-resistant *Plasmodium falciparum* in Pursat province, western Cambodia: a parasite clearance rate study. *Lancet Infect Dis* 2012, 12:851-858.
72. Amaratunga C, Witkowski B, Khim N, Menard D, Fairhurst RM: Artemisinin resistance in *Plasmodium falciparum*. *Lancet Infect Dis* 2014, 14:449-450.
73. Huang F, Takala-Harrison S, Jacob CG, Liu H, Sun X, Yang H, Nyunt MM, Adams M, Zhou S, Xia Z, et al: A Single Mutation in K13 Predominates in Southern China

- and Is Associated With Delayed Clearance of *Plasmodium falciparum* Following Artemisinin Treatment. *J Infect Dis* 2015, 212:1629-1635.
74. Amaratunga C, Witkowski B, Dek D, Try V, Khim N, Miotto O, Menard D, Fairhurst RM: *Plasmodium falciparum* founder populations in western Cambodia have reduced artemisinin sensitivity in vitro. *Antimicrob Agents Chemother* 2014, 58:4935-4937.
 75. Takala-Harrison S, Jacob CG, Arze C, Cummings MP, Silva JC, Dondorp AM, Fukuda MM, Hien TT, Mayxay M, Noedl H, et al: Independent emergence of artemisinin resistance mutations among *Plasmodium falciparum* in Southeast Asia. *J Infect Dis* 2015, 211:670-679.
 76. Imwong M, Suwannasin K, Kunasol C, Sutawong K, Mayxay M, Rekol H, Smithuis FM, Hlaing TM, Tun KM, van der Pluijm RW, et al: The spread of artemisinin-resistant *Plasmodium falciparum* in the Greater Mekong Subregion: a molecular epidemiology observational study. *Lancet Infect Dis* 2017.
 77. Spring MD, Lin JT, Manning JE, Vanachayangkul P, Somethy S, Bun R, Se Y, Chann S, Ittiverakul M, Sia-ngam P, et al: Dihydroartemisinin-piperaquine failure associated with a triple mutant including kelch13 C580Y in Cambodia: an observational cohort study. *Lancet Infect Dis* 2015, 15:683-691.
 78. Duru V, Khim N, Leang R, Kim S, Domergue A, Kloeung N, Ke S, Chy S, Eam R, Khean C, et al: *Plasmodium falciparum* dihydroartemisinin-piperaquine failures in Cambodia are associated with mutant K13 parasites presenting high survival rates in novel piperaquine in vitro assays: retrospective and prospective investigations. *BMC Med* 2015, 13:305.
 79. Amato R, Lim P, Miotto O, Amaratunga C, Dek D, Pearson RD, Almagro-Garcia J, Neal AT, Sreng S, Suon S, et al: Genetic markers associated with dihydroartemisinin-piperaquine failure in *Plasmodium falciparum* malaria in Cambodia: a genotype-phenotype association study. *Lancet Infect Dis* 2016.

80. Conrad MD, Bigira V, Kapisi J, Muhindo M, Kanya MR, Havlir DV, Dorsey G, Rosenthal PJ: Polymorphisms in K13 and falcipain-2 associated with artemisinin resistance are not prevalent in *Plasmodium falciparum* isolated from Ugandan children. *PLoS One* 2014, 9:e105690.
81. Kamau E, Campino S, Amenga-Etego L, Drury E, Ishengoma D, Johnson K, Mumba D, Kekre M, Yavo W, Mead D, et al: K13-propeller polymorphisms in *Plasmodium falciparum* parasites from sub-Saharan Africa. *J Infect Dis* 2015, 211:1352-1355.
82. Mishra N, Prajapati SK, Kaitholia K, Bharti RS, Srivastava B, Phookan S, Anvikar AR, Dev V, Sonal GS, Dhariwal AC, et al: Surveillance of artemisinin resistance in *Plasmodium falciparum* in India using the kelch13 molecular marker. *Antimicrob Agents Chemother* 2015, 59:2548-2553.
83. Van Hong N, Amambua-Ngwa A, Tuan NQ, Cuong do D, Giang NT, Van Dung N, Tinh TT, Van Tien N, Phuc BQ, Duong TT, et al: Severe malaria not responsive to artemisinin derivatives in man returning from Angola to Vietnam. *Emerg Infect Dis* 2014, 20:1199-1202.
84. Taylor SM, Parobek CM, DeConti DK, Kayentao K, Coulibaly SO, Greenwood BM, Tagbor H, Williams J, Bojang K, Njie F, et al: Absence of putative artemisinin resistance mutations among *Plasmodium falciparum* in Sub-Saharan Africa: a molecular epidemiologic study. *J Infect Dis* 2015, 211:680-688.
85. Talisuna AO, Karema C, Ogutu B, Juma E, Logedi J, Nyandigisi A, Mulenga M, Mbacham WF, Roper C, Guerin PJ, et al: Mitigating the threat of artemisinin resistance in Africa: improvement of drug-resistance surveillance and response systems. *Lancet Infect Dis* 2012, 12:888-896.
86. Payne D: Spread of chloroquine resistance in *Plasmodium falciparum*. *Parasitol Today* 1987, 3:241-246.

87. Collins WE, Sullivan JS, Fryauff DJ, Kendall J, Jennings V, Galland GG, Morris CL: Adaptation of a chloroquine-resistant strain of *Plasmodium vivax* from Indonesia to New World monkeys. *Am J Trop Med Hyg* 2000, 62:491-495.
88. Baird JK, Basri H, Purnomo, Bangs MJ, Subianto B, Patchen LC, Hoffman SL: Resistance to chloroquine by *Plasmodium vivax* in Irian Jaya, Indonesia. *Am J Trop Med Hyg* 1991, 44:547-552.
89. Baird JK, Leksana B, Masbar S, Fryauff DJ, Sutanihardja MA, Suradi, Wignall FS, Hoffman SL: Diagnosis of resistance to chloroquine by *Plasmodium vivax*: timing of recurrence and whole blood chloroquine levels. *Am J Trop Med Hyg* 1997, 56:621-626.
90. Baird JK: Chloroquine resistance in *Plasmodium vivax*. *Antimicrob Agents Chemother* 2004, 48:4075-4083.
91. Cooper RD, Milhous WK, Rieckmann KH: The efficacy of WR238605 against the blood stages of a chloroquine resistant strain of *Plasmodium vivax*. *Trans R Soc Trop Med Hyg* 1994, 88:691-692.
92. Rungsihirunrat K, Muhamad P, Chaijaroenkul W, Kuesap J, Na-Bangchang K: *Plasmodium vivax* drug resistance genes; Pvmdr1 and Pvcrt-o polymorphisms in relation to chloroquine sensitivity from a malaria endemic area of Thailand. *Korean J Parasitol* 2015, 53:43-49.
93. Petersen I, Eastman R, Lanzer M: Drug-resistant malaria: molecular mechanisms and implications for public health. *FEBS Lett* 2011, 585:1551-1562.
94. Fernandez-Becerra C, Pinazo MJ, Gonzalez A, Alonso PL, del Portillo HA, Gascon J: Increased expression levels of the pvcrt-o and pvmdr1 genes in a patient with severe *Plasmodium vivax* malaria. *Malar J* 2009, 8:55.
95. Suwanarusk R, Russell B, Chavchich M, Chalfein F, Kenangalem E, Kosaisavee V, Prasetyorini B, Piera KA, Barends M, Brockman A, et al: Chloroquine resistant

- Plasmodium vivax*: in vitro characterisation and association with molecular polymorphisms. *PLoS One* 2007, 2:e1089.
96. Russell B, Chalfein F, Prasetyorini B, Kenangalem E, Piera K, Suwanarusk R, Brockman A, Prayoga P, Sugiarto P, Cheng Q, et al: Determinants of in vitro drug susceptibility testing of *Plasmodium vivax*. *Antimicrob Agents Chemother* 2008, 52:1040-1045.
 97. Suwanarusk R, Chavchich M, Russell B, Jaidee A, Chalfein F, Barends M, Prasetyorini B, Kenangalem E, Piera KA, Lek-Uthai U, et al: Amplification of *pvm-dr1* associated with multidrug-resistant *Plasmodium vivax*. *J Infect Dis* 2008, 198:1558-1564.
 98. Nomura T, Carlton JM, Baird JK, del Portillo HA, Fryauff DJ, Rathore D, Fidock DA, Su X, Collins WE, McCutchan TF, et al: Evidence for different mechanisms of chloroquine resistance in 2 *Plasmodium* species that cause human malaria. *J Infect Dis* 2001, 183:1653-1661.
 99. Chehuan YF, Costa MR, Costa JS, Alecrim MG, Nogueira F, Silveira H, Brasil LW, Melo GC, Monteiro WM, Lacerda MV: In vitro chloroquine resistance for *Plasmodium vivax* isolates from the Western Brazilian Amazon. *Malar J* 2013, 12:226.
 100. Powell RD, Brewer GJ, Degowin RL, Alving AS: Studies on a Strain of Chloroquine-Resistant *Plasmodium Falciparum* from Viet-Nam. *Bull World Health Organ* 1964, 31:379-392.
 101. Erhart A: Malaria control in Vietnam: successes and challenges. University of Antwerpen, Parasitology department, Institute of Tropical Medicine, Antwerpen, Belgium; 2006.
 102. National Malaria Control Programme (NMCP): National guideline for malaria diagnosis and treatment *Ministry of Health Decision 3952/QD- BYT on 9 December 1999 (Vietnamese version)* 1999.

103. National Malaria Control Programme (NMCP): Annual Malaria Report 2013 Hanoi. Vietnam 2013. Internal report.
104. National Institute of Malariology, Parasitology and Entomology (NIMPE), Hanoi, Vietnam: Review of antimalaria drug efficacy over the last 10 years in Vietnam. NIMPE report to WHO. NIMPE Hanoi. Vietnam. 2012. Internal Report.
105. Phuc BQ: Efficacy and safety of dihydroartemisinin-piperaquine for the treatment of uncomplicated *Plasmodium falciparum* malaria in Vietnam in 2014. NIMPE Report to WHO on routine monitoring sentinel sites. NIMPE, Hanoi. Vietnam 2015.
106. Thanh NV, Thuy-Nhien N, Tuyen NT, Tong NT, Nha-Ca NT, Dong LT, Quang HH, Farrar J, Thwaites G, White NJ, et al: Rapid decline in the susceptibility of *Plasmodium falciparum* to dihydroartemisinin-piperaquine in the south of Vietnam. *Malar J* 2017, 16:27.
107. Phan GT, de Vries PJ, Tran BQ, Le HQ, Nguyen NV, Nguyen TV, Heisterkamp SH, Kager PA: Artemisinin or chloroquine for blood stage *Plasmodium vivax* malaria in Vietnam. *Trop Med Int Health* 2002, 7:858-864.
108. Pham VT, Hong NV, Nguyen VV, Louisa M, Baird K, Nguyen XX, Grietens KP, Le XH, Tran TD, Rosanas-Urgell A, et al: Confirmed *Plasmodium vivax* resistance to chloroquine in Central Vietnam. *Antimicrob Agents Chemother* 2015.
109. Thriemer K, Hong NV, Rosanas-Urgell A, Phuc BQ, Ha do M, Pockele E, Guetens P, Van NV, Duong TT, Amambua-Ngwa A, et al: Delayed parasite clearance after treatment with dihydroartemisinin-piperaquine in *Plasmodium falciparum* malaria patients in central Vietnam. *Antimicrob Agents Chemother* 2014, 58:7049-7055.
110. NIMPE. Department of Malaria treatment and research: Report on malaria diagnosis and treatment and antimalaria drugs resistance in 2014 - 2015. Report to NMCP, July, 2015 (Vietnamese version). 2015. Internal Report

111. Morrow M, Nguyen QA, Caruana S, Biggs BA, Doan NH, Nong TT: Pathways to malaria persistence in remote central Vietnam: a mixed-method study of health care and the community. *BMC Public Health* 2009, 9:85.
112. Anh NQ, Hung le X, Thuy HN, Tuy TQ, Caruana SR, Biggs BA, Morrow M: KAP surveys and malaria control in Vietnam: findings and cautions about community research. *Southeast Asian J Trop Med Public Health* 2005, 36:572-577.
113. World Health Organization (WHO): Malaria in the greater Mekong subregion: regional and country profiles. Available from: <http://whothailand.healthrepository.org/handle/123456789/713>. Last accessed 7th Oct 2016. *World Health Organization, New Delhi, India* 2010.
114. World Health Organization (WHO): Guideline for the treatment of Malaria. Available from: <http://www.who.int/malaria/publications/atoz/9789241549127/en/>. Last accessed 12th Dec, 2015. 2015.
115. D'Acremont V, Kilowoko M, Kyungu E, Philipina S, Sangu W, Kahama-Marro J, Lengeler C, Cherpillod P, Kaiser L, Genton B: Beyond malaria--causes of fever in outpatient Tanzanian children. *N Engl J Med* 2014, 370:809-817.
116. Kyabayinze DJ, Tibenderana JK, Odong GW, Rwakimari JB, Counihan H: Operational accuracy and comparative persistent antigenicity of HRP2 rapid diagnostic tests for *Plasmodium falciparum* malaria in a hyperendemic region of Uganda. *Malar J* 2008, 7:221.
117. World Health Organization (WHO): Malaria microscopy quality assurance manual, version 1. Available from: http://www.who.int/malaria/publications/malaria_microscopy_QA_manual.pdf. Last accessed 7th Dec, 2015. 2009.
118. World Health Organization (WHO): Microscopy examination of thick and thin blood smears for identification of malaria parasites. Available from:

- http://www.wpro.who.int/mvp/lab_quality/2096_oms_gmp_sop_08_rev.pdf.
Last accessed 27th November 2016. 2016.
119. World Health Organization (WHO): Malaria parasite counting. Available from: http://www.wpro.who.int/mvp/lab_quality/2096_oms_gmp_sop_09_rev.pdf.
Last accessed 27th November 2016. 2016.
120. Payne D: Use and limitations of light microscopy for diagnosing malaria at the primary health care level. *Bull World Health Organ* 1988, 66:621-626.
121. Tangpukdee N, Duangdee C, Wilairatana P, Krudsood S: Malaria diagnosis: a brief review. *Korean J Parasitol* 2009, 47:93-102.
122. World Health Organization (WHO): Malaria rapid diagnostic test performance: summary results of WHO product testing of malaria RDTs: round 1-6 (2008 - 2015). Available from: http://apps.who.int/iris/bitstream/10665/204119/1/9789241510042_eng.pdf?ua=1. Last accessed 3th Feb, 2016. Geneva 2015.
123. Palmer CJ, Lindo JF, Klaskala WI, Quesada JA, Kaminsky R, Baum MK, Ager AL: Evaluation of the OptiMAL test for rapid diagnosis of *Plasmodium vivax* and *Plasmodium falciparum* malaria. *J Clin Microbiol* 1998, 36:203-206.
124. McCutchan TF, Piper RC, Makler MT: Use of malaria rapid diagnostic test to identify *Plasmodium knowlesi* infection. *Emerg Infect Dis* 2008, 14:1750-1752.
125. Foundation for Innovative New Diagnostics (FIND): Malaria Rapid diagnosis test performance. Results of WHO product testing of malaria RDT round 6 (2014-2015). Available from: http://www.finddx.org/wp-content/uploads/2016/02/WHO-Malaria_rdt_product-testing-results_Round6_eng.pdf. Last access 13 Jul 2016. 2015.
126. Humar A, Ohrt C, Harrington MA, Pillai D, Kain KC: Parasight F test compared with the polymerase chain reaction and microscopy for the diagnosis of

- Plasmodium falciparum* malaria in travelers. *Am J Trop Med Hyg* 1997, 56:44-48.
127. Iqbal J, Sher A, Rab A: *Plasmodium falciparum* histidine-rich protein 2-based immunocapture diagnostic assay for malaria: cross-reactivity with rheumatoid factors. *J Clin Microbiol* 2000, 38:1184-1186.
128. Wongsrichanalai C, Barcus MJ, Muth S, Sutamihardja A, Wernsdorfer WH: A review of malaria diagnostic tools: microscopy and rapid diagnostic test (RDT). *Am J Trop Med Hyg* 2007, 77:119-127.
129. Ndao M: Diagnosis of parasitic diseases: old and new approaches. *Interdiscip Perspect Infect Dis* 2009, 2009:278246.
130. Jacobs J, Barbe B, Gillet P, Aidoo M, Serra-Casas E, Van Erps J, Daviaud J, Incardona S, Cunningham J, Visser T: Harmonization of malaria rapid diagnostic tests: best practices in labelling including instructions for use. *Malar J* 2014, 13:505.
131. Cheng Q, Gatton ML, Barnwell J, Chiodini P, McCarthy J, Bell D, Cunningham J: *Plasmodium falciparum* parasites lacking histidine-rich protein 2 and 3: a review and recommendations for accurate reporting. *Malar J* 2014, 13:283.
132. Kumar N, Singh JP, Pande V, Mishra N, Srivastava B, Kapoor R, Valecha N, Anvikar AR: Genetic variation in histidine rich proteins among Indian *Plasmodium falciparum* population: possible cause of variable sensitivity of malaria rapid diagnostic tests. *Malar J* 2012, 11:298.
133. Li P, Xing H, Zhao Z, Yang Z, Cao Y, Li W, Yan G, Sattabongkot J, Cui L, Fan Q: Genetic diversity of *Plasmodium falciparum* histidine-rich protein 2 in the China-Myanmar border area. *Acta Trop* 2015, 152:26-31.
134. Mouatcho JC, Goldring JP: Malaria rapid diagnostic tests: challenges and prospects. *J Med Microbiol* 2013, 62:1491-1505.

135. Snounou G VS, Zhu XP, Jarra W, Pinheiro L, do Rosario VE, Thaithong S, Brown KN: High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction *Mol Biochem Parasitol* 1993, 61: 315-320.
136. Kamau E, Tolbert LS, Kortepeter L, Pratt M, Nyakoe N, Muringo L, Ogutu B, Waitumbi JN, Ockenhouse CF: Development of a highly sensitive genus-specific quantitative reverse transcriptase real-time PCR assay for detection and quantitation of *plasmodium* by amplifying RNA and DNA of the 18S rRNA genes. *J Clin Microbiol* 2011, 49:2946-2953.
137. Murphy SC, Prentice JL, Williamson K, Wallis CK, Fang FC, Fried M, Pinzon C, Wang R, Talley AK, Kappe SH, et al: Real-time quantitative reverse transcription PCR for monitoring of blood-stage *Plasmodium falciparum* infections in malaria human challenge trials. *Am J Trop Med Hyg* 2012, 86:383-394.
138. World Health Organization (WHO): Malaria diagnosis in low transmission setting Available from: <http://www.who.int/malaria/publications/atoz/policy-brief-diagnosis-low-transmission-settings/en/>. Last accessed 6th Dec, 2015. 2014.
139. Vinetz JM, Gilman RH: Asymptomatic *Plasmodium* parasitemia and the ecology of malaria transmission. *Am J Trop Med Hyg* 2002, 66:639-640.
140. Castelli F CGDom: Diagnosis of malaria infections. . *Health Co-operation papers* 1997, 15: 109-136.
141. Chotivanich K SK, Day NPJ. *Aust J Med Sci*. 2006;27:11–15.: Laboratory diagnosis of malaria infection-a short review of methods. *Aust J Med Sci* 2006, 27:11–15.
142. Shakely D, Elfving K, Aydin-Schmidt B, Msellem MI, Morris U, Omar R, Weiping X, Petzold M, Greenhouse B, Baltzell KA, et al: The usefulness of rapid diagnostic tests in the new context of low malaria transmission in Zanzibar. *PLoS One* 2013, 8:e72912.
143. Rubio JM, Post RJ, van Leeuwen WM, Henry MC, Lindergard G, Hommel M: Alternative polymerase chain reaction method to identify *Plasmodium* species

- in human blood samples: the semi-nested multiplex malaria PCR (SnM-PCR). *Trans R Soc Trop Med Hyg* 2002, 96 Suppl 1:S199-204.
144. Rosanas-Urgell A, Mueller D, Betuela I, Barnadas C, Iga J, Zimmerman PA, del Portillo HA, Siba P, Mueller I, Felger I: Comparison of diagnostic methods for the detection and quantification of the four sympatric *Plasmodium* species in field samples from Papua New Guinea. *Malar J* 2010, 9:361.
 145. Kersting S, Rausch V, Bier FF, von Nickisch-Roseneck M: Rapid detection of *Plasmodium falciparum* with isothermal recombinase polymerase amplification and lateral flow analysis. *Malar J* 2014, 13:99.
 146. Lucchi NW, Narayanan J, Karell MA, Xayavong M, Kariuki S, DaSilva AJ, Hill V, Udhayakumar V: Molecular diagnosis of malaria by photo-induced electron transfer fluorogenic primers: PET-PCR. *PLoS One* 2013, 8:e56677.
 147. Safeukui I, Millet P, Boucher S, Melinard L, Fregeville F, Receveur MC, Pistone T, Fialon P, Vincendeau P, Fleury H, Malvy D: Evaluation of FRET real-time PCR assay for rapid detection and differentiation of *Plasmodium* species in returning travellers and migrants. *Malar J* 2008, 7:70.
 148. Talundzic E, Maganga M, Masanja IM, Peterson DS, Udhayakumar V, Lucchi NW: Field evaluation of the photo-induced electron transfer fluorogenic primers (PET) real-time PCR for the detection of *Plasmodium falciparum* in Tanzania. *Malar J* 2014, 13:31.
 149. Cheng Z, Sun X, Yang Y, Wang H, Zheng Z: A novel, sensitive assay for high-throughput molecular detection of plasmodia for active screening of malaria for elimination. *J Clin Microbiol* 2013, 51:125-130.
 150. Schoone GJ, Oskam L, Kroon NC, Schallig HD, Omar SA: Detection and quantification of *Plasmodium falciparum* in blood samples using quantitative nucleic acid sequence-based amplification. *J Clin Microbiol* 2000, 38:4072-4075.

151. Hofmann N, Mwingira F, Shekalaghe S, Robinson LJ, Mueller I, Felger I: Ultra-sensitive detection of *Plasmodium falciparum* by amplification of multi-copy subtelomeric targets. *PLoS Med* 2015, 12:e1001788.
152. Swan H, Sloan L, Muyombwe A, Chavalitshewinkoon-Petmitr P, Krudsood S, Leowattana W, Wilairatana P, Looareesuwan S, Rosenblatt J: Evaluation of a real-time polymerase chain reaction assay for the diagnosis of malaria in patients from Thailand. *Am J Trop Med Hyg* 2005, 73:850-854.
153. Mens PF, Schoone GJ, Kager PA, Schallig HD: Detection and identification of human *Plasmodium* species with real-time quantitative nucleic acid sequence-based amplification. *Malar J* 2006, 5:80.
154. Singh B, Daneshvar C: Human infections and detection of *Plasmodium knowlesi*. *Clin Microbiol Rev* 2013, 26:165-184.
155. Farrugia C, Cabaret O, Botterel F, Bories C, Foulet F, Costa JM, Bretagne S: Cytochrome b gene quantitative PCR for diagnosing *Plasmodium falciparum* infection in travelers. *J Clin Microbiol* 2011, 49:2191-2195.
156. Campino S, Auburn S, Kivinen K, Zongo I, Ouedraogo JB, Mangano V, Djimde A, Doumbo OK, Kiara SM, Nzila A, et al: Population genetic analysis of *Plasmodium falciparum* parasites using a customized Illumina GoldenGate genotyping assay. *PLoS One* 2011, 6:e20251.
157. Polley SD, Mori Y, Watson J, Perkins MD, Gonzalez IJ, Notomi T, Chiodini PL, Sutherland CJ: Mitochondrial DNA targets increase sensitivity of malaria detection using loop-mediated isothermal amplification. *J Clin Microbiol* 2010, 48:2866-2871.
158. Berry A, Deymier C, Sertorio M, Witkowski B, Benoit-Vical F: Pfs 16 pivotal role in *Plasmodium falciparum* gametocytogenesis: a potential antiparasitic drug target. *Exp Parasitol* 2009, 121:189-192.

159. Cheng Q, Cloonan N, Fischer K, Thompson J, Waine G, Lanzer M, Saul A: *stevor* and *rif* are *Plasmodium falciparum* multicopy gene families which potentially encode variant antigens. *Mol Biochem Parasitol* 1998, 97:161-176.
160. Wampfler R, Mwingira F, Javati S, Robinson L, Betuela I, Siba P, Beck HP, Mueller I, Felger I: Strategies for detection of *Plasmodium* species gametocytes. *PLoS One* 2013, 8:e76316.
161. Canier L, Khim N, Kim S, Eam R, Khean C, Loch K, Ken M, Pannus P, Bosman P, Stassijns J, et al: Malaria PCR detection in Cambodian low-transmission settings: dried blood spots versus venous blood samples. *Am J Trop Med Hyg* 2015, 92:573-577.
162. Ataei S, Nateghpour M, Hajjarian H, Edrissian GH, Foroushani AR: High specificity of semi-nested multiplex PCR using dried blood spots on DNA Banking Card in comparison with frozen liquid blood for detection of *Plasmodium falciparum* and *Plasmodium vivax*. *J Clin Lab Anal* 2011, 25:185-190.
163. Strom GE, Moyo S, Fataki M, Langeland N, Blomberg B: PCR targeting *Plasmodium* mitochondrial genome of DNA extracted from dried blood on filter paper compared to whole blood. *Malar J* 2014, 13:137.
164. Taylor SM, Juliano JJ, Trottman PA, Griffin JB, Landis SH, Kitsa P, Tshefu AK, Meshnick SR: High-throughput pooling and real-time PCR-based strategy for malaria detection. *J Clin Microbiol* 2010, 48:512-519.
165. Champlot S, Berthelot C, Pruvost M, Bennett EA, Grange T, Geigl EM: An efficient multistrategy DNA decontamination procedure of PCR reagents for hypersensitive PCR applications. *PLoS One* 2010, 5.
166. Bousema T, Okell L, Felger I, Drakeley C: Asymptomatic malaria infections: detectability, transmissibility and public health relevance. *Nat Rev Microbiol* 2014, 12:833-840.

167. Karl S, Laman M, Koleala T, Ibam C, Kasian B, N'Drewei N, Rosanas-Urgell A, Moore BR, Waltmann A, Koepfli C, et al: Comparison of three methods for detection of gametocytes in Melanesian children treated for uncomplicated malaria. *Malar J* 2014, 13:319.
168. Schneider P, Schoone G, Schallig H, Verhage D, Telgt D, Eling W, Sauerwein R: Quantification of *Plasmodium falciparum* gametocytes in differential stages of development by quantitative nucleic acid sequence-based amplification. *Mol Biochem Parasitol* 2004, 137:35-41.
169. Beurskens M, Mens P, Schallig H, Syafruddin D, Asih PB, Hermsen R, Sauerwein R: Quantitative determination of *Plasmodium vivax* gametocytes by real-time quantitative nucleic acid sequence-based amplification in clinical samples. *Am J Trop Med Hyg* 2009, 81:366-369.
170. Buates S, Bantuchai S, Sattabongkot J, Han ET, Tsuboi T, Udomsangpetch R, Sirichaisinthop J, Tan-ariya P: Development of a reverse transcription-loop-mediated isothermal amplification (RT-LAMP) for clinical detection of *Plasmodium falciparum* gametocytes. *Parasitol Int* 2010, 59:414-420.
171. Kast K, Berens-Riha N, Zeynudin A, Abduselam N, Eshetu T, Loscher T, Wieser A, Shock J, Pritsch M: Evaluation of *Plasmodium falciparum* gametocyte detection in different patient material. *Malar J* 2013, 12:438.
172. Abdul-Ghani R, Beier JC: Strategic use of antimalarial drugs that block falciparum malaria parasite transmission to mosquitoes to achieve local malaria elimination. *Parasitol Res* 2014, 113:3535-3546.
173. Abdul-Ghani R, Farag HF, Allam AF, Azazy AA: Measuring resistant-genotype transmission of malaria parasites: challenges and prospects. *Parasitol Res* 2014, 113:1481-1487.

174. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T: Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res* 2000, 28:E63.
175. Poon LL, Wong BW, Ma EH, Chan KH, Chow LM, Abeyewickreme W, Tangpukdee N, Yuen KY, Guan Y, Looareesuwan S, Peiris JS: Sensitive and inexpensive molecular test for falciparum malaria: detecting *Plasmodium falciparum* DNA directly from heat-treated blood by loop-mediated isothermal amplification. *Clin Chem* 2006, 52:303-306.
176. Han ET, Watanabe R, Sattabongkot J, Khuntirat B, Sirichaisinthop J, Iriko H, Jin L, Takeo S, Tsuboi T: Detection of four *Plasmodium* species by genus- and species-specific loop-mediated isothermal amplification for clinical diagnosis. *J Clin Microbiol* 2007, 45:2521-2528.
177. Polley SD, Gonzalez IJ, Mohamed D, Daly R, Bowers K, Watson J, Mewse E, Armstrong M, Gray C, Perkins MD, et al: Clinical evaluation of a loop-mediated amplification kit for diagnosis of imported malaria. *J Infect Dis* 2013, 208:637-644.
178. Hopkins H, Gonzalez IJ, Polley SD, Angutoko P, Ategeka J, Asiimwe C, Agaba B, Kyabayinze DJ, Sutherland CJ, Perkins MD, Bell D: Highly sensitive detection of malaria parasitemia in a malaria-endemic setting: performance of a new loop-mediated isothermal amplification kit in a remote clinic in Uganda. *J Infect Dis* 2013, 208:645-652.
179. Aydin-Schmidt B, Xu W, Gonzalez IJ, Polley SD, Bell D, Shakely D, Msellem MI, Bjorkman A, Martensson A: Loop mediated isothermal amplification (LAMP) accurately detects malaria DNA from filter paper blood samples of low density parasitaemias. *PLoS One* 2014, 9:e103905.
180. Vallejo AF, Martinez NL, Gonzalez IJ, Arevalo-Herrera M, Herrera S: Evaluation of the loop mediated isothermal DNA amplification (LAMP) kit for malaria

- diagnosis in *P. vivax* endemic settings of Colombia. *PLoS Negl Trop Dis* 2015, 9:e3453.
181. Sattabongkot J, Tsuboi T, Han ET, Bantuchai S, Buates S: Loop-mediated isothermal amplification assay for rapid diagnosis of malaria infections in an area of endemicity in Thailand. *J Clin Microbiol* 2014, 52:1471-1477.
 182. Lau YL, Fong MY, Mahmud R, Chang PY, Palaeya V, Cheong FW, Chin LC, Anthony CN, Al-Mekhlafi AM, Chen Y: Specific, sensitive and rapid detection of human *plasmodium knowlesi* infection by loop-mediated isothermal amplification (LAMP) in blood samples. *Malar J* 2011, 10:197.
 183. Lau YL, Lai MY, Fong MY, Jelip J, Mahmud R: Loop-Mediated Isothermal Amplification Assay for Identification of Five Human *Plasmodium* Species in Malaysia. *Am J Trop Med Hyg* 2016, 94:336-339.
 184. Tomita N, Mori Y, Kanda H, Notomi T: Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. *Nat Protoc* 2008, 3:877-882.
 185. Notomi T, Mori Y, Tomita N, Kanda H: Loop-mediated isothermal amplification (LAMP): principle, features, and future prospects. *J Microbiol* 2015, 53:1-5.
 186. Han ET: Loop-mediated isothermal amplification test for the molecular diagnosis of malaria. *Expert Rev Mol Diagn* 2013, 13:205-218.
 187. Oriero EC, Okebe J, Jacobs J, Van Geertruyden JP, Nwakanma D, D'Alessandro U: Diagnostic performance of a novel loop-mediated isothermal amplification (LAMP) assay targeting the apicoplast genome for malaria diagnosis in a field setting in sub-Saharan Africa. *Malar J* 2015, 14:396.
 188. Dhama K, Karthik K, Chakraborty S, Tiwari R, Kapoor S, Kumar A, Thomas P: Loop-mediated isothermal amplification of DNA (LAMP): a new diagnostic tool lights the world of diagnosis of animal and human pathogens: a review. *Pak J Biol Sci* 2014, 17:151-166.

189. Hsiang MS, Greenhouse B, Rosenthal PJ: Point of care testing for malaria using LAMP, loop mediated isothermal amplification. *J Infect Dis* 2014, 210:1167-1169.
190. Cook J, Aydin-Schmidt B, Gonzalez IJ, Bell D, Edlund E, Nasser MH, Msellem M, Ali A, Abass AK, Martensson A, Bjorkman A: Loop-mediated isothermal amplification (LAMP) for point-of-care detection of asymptomatic low-density malaria parasite carriers in Zanzibar. *Malar J* 2015, 14:43.
191. Barry AE, Waltmann A, Koepfli C, Barnadas C, Mueller I: Uncovering the transmission dynamics of *Plasmodium vivax* using population genetics. *Pathog Glob Health* 2015, 109:142-152.
192. Koepfli C, Rodrigues PT, Antao T, Orjuela-Sanchez P, Van den Eede P, Gamboa D, van Hong N, Bendezu J, Erhart A, Barnadas C, et al: *Plasmodium vivax* Diversity and Population Structure across Four Continents. *PLoS Negl Trop Dis* 2015, 9:e0003872.
193. Wei G, Zhang L, Yan H, Zhao Y, Hu J, Pan W: Evaluation of the population structure and genetic diversity of *Plasmodium falciparum* in southern China. *Malar J* 2015, 14:283.
194. Sutton PL: A call to arms: on refining *Plasmodium vivax* microsatellite marker panels for comparing global diversity. *Malar J* 2013, 12:447.
195. Su X, Hayton K, Wellems TE: Genetic linkage and association analyses for trait mapping in *Plasmodium falciparum*. *Nat Rev Genet* 2007, 8:497-506.
196. Koepfli C, Mueller I, Marfurt J, Goroti M, Sie A, Oa O, Genton B, Beck HP, Felger I: Evaluation of *Plasmodium vivax* genotyping markers for molecular monitoring in clinical trials. *J Infect Dis* 2009, 199:1074-1080.
197. Kolakovich KA, Ssengoba A, Wojcik K, Tsuboi T, al-Yaman F, Alpers M, Adams JH: *Plasmodium vivax*: favored gene frequencies of the merozoite surface protein-

- 1 and the multiplicity of infection in a malaria endemic region. *Exp Parasitol* 1996, 83:11-19.
198. Bruce MC, Galinski MR, Barnwell JW, Donnelly CA, Walmsley M, Alpers MP, Walliker D, Day KP: Genetic diversity and dynamics of *plasmodium falciparum* and *P. vivax* populations in multiply infected children with asymptomatic malaria infections in Papua New Guinea. *Parasitology* 2000, 121 (Pt 3):257-272.
 199. Barry AE, Schultz L, Buckee CO, Reeder JC: Contrasting population structures of the genes encoding ten leading vaccine-candidate antigens of the human malaria parasite, *Plasmodium falciparum*. *PLoS One* 2009, 4:e8497.
 200. Craig AA, Kain KC: Molecular analysis of strains of *Plasmodium vivax* from paired primary and relapse infections. *J Infect Dis* 1996, 174:373-379.
 201. Koepfli C, Colborn KL, Kiniboro B, Lin E, Speed TP, Siba PM, Felger I, Mueller I: A high force of *plasmodium vivax* blood-stage infection drives the rapid acquisition of immunity in papua new guinean children. *PLoS Negl Trop Dis* 2013, 7:e2403.
 202. Mueller I, Schoepflin S, Smith TA, Benton KL, Bretscher MT, Lin E, Kiniboro B, Zimmerman PA, Speed TP, Siba P, Felger I: Force of infection is key to understanding the epidemiology of *Plasmodium falciparum* malaria in Papua New Guinean children. *Proc Natl Acad Sci U S A* 2012, 109:10030-10035.
 203. Takala SL, Escalante AA, Branch OH, Kariuki S, Biswas S, Chaiyaroj SC, Lal AA: Genetic diversity in the Block 2 region of the merozoite surface protein 1 (MSP-1) of *Plasmodium falciparum*: additional complexity and selection and convergence in fragment size polymorphism. *Infect Genet Evol* 2006, 6:417-424.
 204. Strand M, Prolla TA, Liskay RM, Petes TD: Destabilization of tracts of simple repetitive DNA in yeast by mutations affecting DNA mismatch repair. *Nature* 1993, 365:274-276.

205. Ajzenberg D, Banuls AL, Tibayrenc M, Darde ML: Microsatellite analysis of *Toxoplasma gondii* shows considerable polymorphism structured into two main clonal groups. *Int J Parasitol* 2002, 32:27-38.
206. Montoya L, Gallego M, Gavignet B, Piarroux R, Rioux JA, Portus M, Fisa R: Application of microsatellite genotyping to the study of a restricted *Leishmania infantum* focus: different genotype compositions in isolates from dogs and sand flies. *Am J Trop Med Hyg* 2007, 76:888-895.
207. Anderson TJ, Haubold B, Williams JT, Estrada-Franco JG, Richardson L, Mollinedo R, Bockarie M, Mokili J, Mharakurwa S, French N, et al: Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium falciparum*. *Mol Biol Evol* 2000, 17:1467-1482.
208. Imwong M, Nair S, Pukrittayakamee S, Sudimack D, Williams JT, Mayxay M, Newton PN, Kim JR, Nandy A, Osorio L, et al: Contrasting genetic structure in *Plasmodium vivax* populations from Asia and South America. *Int J Parasitol* 2007, 37:1013-1022.
209. Carlton JM, Adams JH, Silva JC, Bidwell SL, Lorenzi H, Caler E, Crabtree J, Angiuoli SV, Merino EF, Amedeo P, et al: Comparative genomics of the neglected human malaria parasite *Plasmodium vivax*. *Nature* 2008, 455:757-763.
210. Bruce MC, Macheso A, Galinski MR, Barnwell JW: Characterization and application of multiple genetic markers for *Plasmodium malariae*. *Parasitology* 2007, 134:637-650.
211. Tyagi S, Sharma M, Das A: Comparative genomic analysis of simple sequence repeats in three *Plasmodium* species. *Parasitol Res* 2011, 108:451-458.
212. Su X, Wellems TE: Toward a high-resolution *Plasmodium falciparum* linkage map: polymorphic markers from hundreds of simple sequence repeats. *Genomics* 1996, 33:430-444.

213. Carlton J: The *Plasmodium vivax* genome sequencing project. *Trends Parasitol* 2003, 19:227-231.
214. Gardner MJ, Hall N, Fung E, White O, Berriman M, Hyman RW, Carlton JM, Pain A, Nelson KE, Bowman S, et al: Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* 2002, 419:498-511.
215. Anderson TJ, Su XZ, Bockarie M, Lagog M, Day KP: Twelve microsatellite markers for characterization of *Plasmodium falciparum* from finger-prick blood samples. *Parasitology* 1999, 119 (Pt 2):113-125.
216. Schultz L, Wapling J, Mueller I, Ntsuke PO, Senn N, Nale J, Kiniboro B, Buckee CO, Tavul L, Siba PM, et al: Multilocus haplotypes reveal variable levels of diversity and population structure of *Plasmodium falciparum* in Papua New Guinea, a region of intense perennial transmission. *Malar J* 2010, 9:336.
217. Iwagami M, Rivera PT, Villacorte EA, Escueta AD, Hatabu T, Kawazu S, Hayakawa T, Tanabe K, Kano S: Genetic diversity and population structure of *Plasmodium falciparum* in the Philippines. *Malar J* 2009, 8:96.
218. Carter TE, Malloy H, Existe A, Memnon G, St Victor Y, Okech BA, Mulligan CJ: Genetic Diversity of *Plasmodium falciparum* in Haiti: Insights from Microsatellite Markers. *PLoS One* 2015, 10:e0140416.
219. Noviyanti R, Coutrier F, Utami RA, Trimarsanto H, Tirta YK, Trianty L, Kusuma A, Sutanto I, Kosasih A, Kusriastuti R, et al: Contrasting Transmission Dynamics of Co-endemic *Plasmodium vivax* and *P. falciparum*: Implications for Malaria Control and Elimination. *PLoS Negl Trop Dis* 2015, 9:e0003739.
220. Okombo J, Kamau AW, Marsh K, Sutherland CJ, Ochola-Oyier LI: Temporal trends in prevalence of *Plasmodium falciparum* drug resistance alleles over two decades of changing antimalarial policy in coastal Kenya. *Int J Parasitol Drugs Drug Resist* 2014, 4:152-163.

221. Abdullah NR, Barber BE, William T, Norahmad NA, Satsu UR, Muniandy PK, Ismail Z, Grigg MJ, Jelip J, Piera K, et al: *Plasmodium vivax* population structure and transmission dynamics in Sabah Malaysia. *PLoS One* 2013, 8:e82553.
222. Delgado-Ratto C, Soto-Calle VE, Van den Eede P, Gamboa D, Rosas A, Abatih EN, Rodriguez Ferrucci H, Llanos-Cuentas A, Van Geertruyden JP, Erhart A, D'Alessandro U: Population structure and spatio-temporal transmission dynamics of *Plasmodium vivax* after radical cure treatment in a rural village of the Peruvian Amazon. *Malar J* 2014, 13:8.
223. 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.
224. Honma H, Kim JY, Palacpac NM, Mita T, Lee W, Horii T, Tanabe K: Recent increase of genetic diversity in *Plasmodium vivax* population in the Republic of Korea. *Malar J* 2011, 10:257.
225. Neafsey DE, Galinsky K, Jiang RH, Young L, Sykes SM, Saif S, Gujja S, Goldberg JM, Young S, Zeng Q, et al: The malaria parasite *Plasmodium vivax* exhibits greater genetic diversity than *Plasmodium falciparum*. *Nat Genet* 2012, 44:1046-1050.
226. Ferreira MU, Karunaweera ND, da Silva-Nunes M, da Silva NS, Wirth DF, Hartl DL: Population structure and transmission dynamics of *Plasmodium vivax* in rural Amazonia. *J Infect Dis* 2007, 195:1218-1226.
227. Koepfli C, Timinao L, Antao T, Barry AE, Siba P, Mueller I, Felger I: A Large Reservoir and Little Population Structure in the South Pacific. *PLoS One* 2013, 8:e66041.
228. Van den Eede P, Erhart A, Van der Auwera G, Van Overmeir C, Thang ND, Hung le X, Anne J, D'Alessandro U: High complexity of *Plasmodium vivax* infections in

- symptomatic patients from a rural community in central Vietnam detected by microsatellite genotyping. *Am J Trop Med Hyg* 2010, 82:223-227.
229. Thanapongpichat S, McGready R, Luxemburger C, Day NP, White NJ, Nosten F, Snounou G, Imwong M: Microsatellite genotyping of *Plasmodium vivax* infections and their relapses in pregnant and non-pregnant patients on the Thai-Myanmar border. *Malar J* 2013, 12:275.
230. Beck HP, Wampfler R, Carter N, Koh G, Osorio L, Rueangweerayut R, Krudsood S, Lacerda MV, Llanos-Cuentas A, Duparc S, et al: Estimation of the Antirelapse Efficacy of Tafenoquine, Using *Plasmodium vivax* Genotyping. *J Infect Dis* 2016, 213:794-799.
231. Manrique P, Hoshi M, Fasabi M, Nolasco O, Yori P, Calderon M, Gilman RH, Kosek MN, Vinetz JM, Gamboa D: Assessment of an automated capillary system for *Plasmodium vivax* microsatellite genotyping. *Malar J* 2015, 14:326.
232. Iwagami M, Hwang SY, Kim SH, Park SJ, Lee GY, Matsumoto-Takahashi EL, Kho WG, Kano S: Microsatellite DNA analysis revealed a drastic genetic change of *Plasmodium vivax* population in the Republic of Korea during 2002 and 2003. *PLoS Negl Trop Dis* 2013, 7:e2522.
233. 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.
234. Yalcindag E, Elguero E, Arnathau C, Durand P, Akiana J, Anderson TJ, Aubouy A, Balloux F, Besnard P, Bogreau H, et al: Multiple independent introductions of *Plasmodium falciparum* in South America. *Proc Natl Acad Sci U S A* 2012, 109:511-516.
235. Fernandez ME, Goszczynski DE, Liron JP, Villegas-Castagnasso EE, Carino MH, Ripoli MV, Rogberg-Munoz A, Posik DM, Peral-Garcia P, Giovambattista G:

- Comparison of the effectiveness of microsatellites and SNP panels for genetic identification, traceability and assessment of parentage in an inbred Angus herd. *Genet Mol Biol* 2013, 36:185-191.
236. Daniels R, Volkman SK, Milner DA, Mahesh N, Neafsey DE, Park DJ, Rosen D, Angelino E, Sabeti PC, Wirth DF, Wiegand RC: A general SNP-based molecular barcode for *Plasmodium falciparum* identification and tracking. *Malar J* 2008, 7:223.
237. Volkman SK, Sabeti PC, DeCaprio D, Neafsey DE, Schaffner SF, Milner DA, Jr., Daily JP, Sarr O, Ndiaye D, Ndir O, et al: A genome-wide map of diversity in *Plasmodium falciparum*. *Nat Genet* 2007, 39:113-119.
238. Baniecki ML, Faust AL, Schaffner SF, Park DJ, Galinsky K, Daniels RF, Hamilton E, Ferreira MU, Karunaweera ND, Serre D, et al: Development of a single nucleotide polymorphism barcode to genotype *Plasmodium vivax* infections. *PLoS Negl Trop Dis* 2015, 9:e0003539.
239. Preston MD, Campino S, Assefa SA, Echeverry DF, Ocholla H, Amambua-Ngwa A, Stewart LB, Conway DJ, Borrmann S, Michon P, et al: A barcode of organellar genome polymorphisms identifies the geographic origin of *Plasmodium falciparum* strains. *Nat Commun* 2014, 5:4052.
240. Carlton JM, Volkman SK, Uplekar S, Hupalo DN, Pereira Alves JM, Cui L, Donnelly M, Roos DS, Harb OS, Acosta M, et al: Population Genetics, Evolutionary Genomics, and Genome-Wide Studies of Malaria: A View Across the International Centers of Excellence for Malaria Research. *Am J Trop Med Hyg* 2015, 93:87-98.
241. Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ: High-resolution genotyping by amplicon melting analysis using LCGreen. *Clin Chem* 2003, 49:853-860.

242. Daniels R, Chang HH, Sene PD, Park DC, Neafsey DE, Schaffner SF, Hamilton EJ, Lukens AK, Van Tyne D, Mboup S, et al: Genetic surveillance detects both clonal and epidemic transmission of malaria following enhanced intervention in Senegal. *PLoS One* 2013, 8:e60780.
243. Lachance J, Tishkoff SA: SNP ascertainment bias in population genetic analyses: why it is important, and how to correct it. *Bioessays* 2013, 35:780-786.
244. Haasl RJ, Payseur BA: Multi-locus inference of population structure: a comparison between single nucleotide polymorphisms and microsatellites. *Heredity (Edinb)* 2011, 106:158-171.
245. Conway DJ: Molecular epidemiology of malaria. *Clin Microbiol Rev* 2007, 20:188-204.
246. Escalante AA, Ferreira MU, Vinetz JM, Volkman SK, Cui L, Gamboa D, Krogstad DJ, Barry AE, Carlton JM, van Eijk AM, et al: Malaria Molecular Epidemiology: Lessons from the International Centers of Excellence for Malaria Research Network. *Am J Trop Med Hyg* 2015, 93:79-86.
247. Vinayak S, Alam MT, Sem R, Shah NK, Susanti AI, Lim P, Muth S, Maguire JD, Rogers WO, Fandeur T, et al: Multiple Genetic Backgrounds of the Amplified *Plasmodium falciparum* Multidrug Resistance (pfmdr1) Gene and Selective Sweep of 184F Mutation in Cambodia. *Journal of Infectious Diseases* 2010, 201:1551-1560.
248. Kerlin DH, Boyce K, Marfurt J, Simpson JA, Kenangalem E, Cheng Q, Price RN, Gatton ML: An analytical method for assessing stage-specific drug activity in *Plasmodium vivax* malaria: implications for ex vivo drug susceptibility testing. *PLoS Negl Trop Dis* 2012, 6:e1772.
249. Basco LK: Field application of in vitro assays for the sensitivity of human malaria parasites to antimalarial drug. Available from:

http://whqlibdoc.who.int/publications/2007/9789241595155_eng.pdf. Last accessed 22 May 2015. . WHO 2010.

250. Oyola SO, Manske M, Campino S, Claessens A, Hamilton WL, Kekre M, Drury E, Mead D, Gu Y, Miles A, et al: Optimized whole-genome amplification strategy for extremely AT-biased template. *DNA Res* 2014, 21:661-671.
251. Bright AT, Tewhey R, Abeles S, Chuquiyaui R, Llanos-Cuentas A, Ferreira MU, Schork NJ, Vinetz JM, Winzeler EA: Whole genome sequencing analysis of *Plasmodium vivax* using whole genome capture. *BMC Genomics* 2012, 13:262.
252. Ball AD, Stapley J, Dawson DA, Birkhead TR, Burke T, Slate J: A comparison of SNPs and microsatellites as linkage mapping markers: lessons from the zebra finch (*Taeniopygia guttata*). *BMC Genomics* 2010, 11:218.
253. Doderer C, Heschung A, Guntz P, Cazenave JP, Hansmann Y, Senegas A, Pfaff AW, Abdelrahman T, Candolfi E: A new ELISA kit which uses a combination of *Plasmodium falciparum* extract and recombinant *Plasmodium vivax* antigens as an alternative to IFAT for detection of malaria antibodies. *Malar J* 2007, 6:19.
254. Doolan DL, Dobano C, Baird JK: Acquired immunity to malaria. *Clin Microbiol Rev* 2009, 22:13-36, Table of Contents.
255. Tusting LS, Bousema T, Smith DL, Drakeley C: Measuring changes in *Plasmodium falciparum* transmission: precision, accuracy and costs of metrics. *Adv Parasitol* 2014, 84:151-208.
256. Bosomprah S: A mathematical model of seropositivity to malaria antigen, allowing seropositivity to be prolonged by exposure. *Malar J* 2014, 13:12.
257. van den Hoogen LL, Griffin JT, Cook J, Sepulveda N, Corran P, Conway DJ, Milligan P, Affara M, Allen SJ, Proietti C, et al: Serology describes a profile of declining malaria transmission in Farafenni, The Gambia. *Malar J* 2015, 14:416.
258. Cook J, Speybroeck N, Sochantá 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.
259. Elliott SR, Fowkes FJ, Richards JS, Reiling L, Drew DR, Beeson JG: Research priorities for the development and implementation of serological tools for malaria surveillance. *F1000Prime Rep* 2014, 6:100.
 260. Kim TS, Kang YJ, Lee WJ, Na BK, Moon SU, Cha SH, Lee SK, Park YK, Pak JH, Cho PY, et al: Seroprevalence of *Plasmodium vivax* in the Republic of Korea (2003-2005) using indirect fluorescent antibody test. *Korean J Parasitol* 2014, 52:1-7.
 261. Candolfi E: Transfusion-transmitted malaria, preventive measures. *Transfus Clin Biol* 2005, 12:107-113.
 262. Noedl H, Yingyuen K, Laoboonchai A, Fukuda M, Sirichaisinthop J, Miller RS: Sensitivity and specificity of an antigen detection ELISA for malaria diagnosis. *Am J Trop Med Hyg* 2006, 75:1205-1208.
 263. Priest JW, Jenks MH, Moss DM, Mao B, Buth S, Wannemuehler K, Soeung SC, Lucchi NW, Udhayakumar V, Gregory CJ, et al: Integration of Multiplex Bead Assays for Parasitic Diseases into a National, Population-Based Serosurvey of Women 15-39 Years of Age in Cambodia. *PLoS Negl Trop Dis* 2016, 10:e0004699.
 264. Kerkhof K, Canier L, Kim S, Heng S, Sochantha T, Sovannaroth S, Vigan-Womas I, Coosemans M, Sluydts V, Menard D, Durnez L: Implementation and application of a multiplex assay to detect malaria-specific antibodies: a promising tool for assessing malaria transmission in Southeast Asian pre-elimination areas. *Malar J* 2015, 14:338.
 265. Ambrosino E, Dumoulin C, Orlandi-Pradines E, Remoue F, Toure-Balde A, Tall A, Sarr JB, Poinsignon A, Sokhna C, Puget K, et al: A multiplex assay for the simultaneous detection of antibodies against 15 *Plasmodium falciparum* and *Anopheles gambiae* saliva antigens. *Malar J* 2010, 9:317.

266. Koffi D, Toure AO, Varela ML, Vigan-Womas I, Beourou S, Brou S, Ehouman MF, Gnamien L, Richard V, Djaman JA, Perraut R: Analysis of antibody profiles in symptomatic malaria in three sentinel sites of Ivory Coast by using multiplex, fluorescent, magnetic, bead-based serological assay (MAGPIX). *Malar J* 2015, 14:509.
267. Elshal MF, McCoy JP: Multiplex bead array assays: performance evaluation and comparison of sensitivity to ELISA. *Methods* 2006, 38:317-323.
268. Baum E, Badu K, Molina DM, Liang X, Felgner PL, Yan G: Protein microarray analysis of antibody responses to *Plasmodium falciparum* in western Kenyan highland sites with differing transmission levels. *PLoS One* 2013, 8:e82246.
269. Fouda GG, Leke RF, Long C, Druilhe P, Zhou A, Taylor DW, Johnson AH: Multiplex assay for simultaneous measurement of antibodies to multiple *Plasmodium falciparum* antigens. *Clin Vaccine Immunol* 2006, 13:1307-1313.
270. Sturrock HJ, Hsiang MS, Cohen JM, Smith DL, Greenhouse B, Bousema T, Gosling RD: Targeting asymptomatic malaria infections: active surveillance in control and elimination. *PLoS Med* 2013, 10:e1001467.
271. 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.
272. Thang ND, Erhart A, Speybroeck N, Hung le X, Thuan le K, Hung CT, Ky PV, Coosemans M, D'Alessandro U: Malaria in central Vietnam: analysis of risk factors by multivariate analysis and classification tree models. *Malar J* 2008, 7:28.
273. Erhart A, Ngo DT, Phan VK, Ta TT, Van Overmeir C, Speybroeck N, Obsomer V, Le XH, Le KT, Coosemans M, D'Alessandro U: Epidemiology of forest malaria in central Vietnam: a large scale cross-sectional survey. *Malar J* 2005, 4:58.

274. Erhart A, Thang ND, Bien TH, Tung NM, Hung NQ, Hung LX, Tuy TQ, Speybroeck N, Cong LD, Coosemans M, D'Alessandro U: Malaria epidemiology in a rural area of the Mekong Delta: a prospective community-based study. *Trop Med Int Health* 2004, 9:1081-1090.
275. Erhart A, Thang ND, Hung NQ, Toi le V, Hung le X, Tuy TQ, Cong le D, Speybroeck N, Coosemans M, D'Alessandro U: Forest malaria in Vietnam: a challenge for control. *Am J Trop Med Hyg* 2004, 70:110-118.

Chapter 3

Short report: A modified semi-nested multiplex malaria PCR (SnM – PCR) for the identification of the five human *Plasmodium* species occurring in Southeast Asia

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Abstract

We have modified an existing semi-nested multiplex polymerase chain reaction (PCR) by adding one *Plasmodium knowlesi*-specific nested PCR, and validated the latter against laboratory and clinical samples. This new method has the advantage of being relatively affordable in low resource settings while identifying the five human *Plasmodium* species with a three-step PCR.

The study

Since the first Malaysian reports and the recognition of *Plasmodium knowlesi* as the fifth human *Plasmodium* species [1-5], there have been continuous attempts to improve the molecular detection of this *Plasmodium* species of simian origin [6-11]. Initially, a nested 18S small subunit (ssu) ribosomal DNA (rDNA) based polymerase chain reaction (PCR) identified the first large cohort of naturally acquired *P. knowlesi* infections in humans in Borneo [1]. However, the primers used to detect the *P. knowlesi* DNA (Pmk8-Pmkr9) can cross react with the *P. vivax* ribosomal RNA (rRNA) gene, requiring systematic confirmation by either a secondary PCR [1] or sequencing [6,12]. Since then, more specific methods using nested [6] or non-nested PCR [10, 11], loop mediated isothermal amplification [8], or real time PCR [7,9] have been developed.

Plasmodium knowlesi infections in humans were reported in Vietnam [12] and this species needs to be accounted for in the newly defined national malaria elimination strategies [13]. Applying the multiplex semi-nested (SnM) PCR published by Rubio and others [14], for the detection of the four previously described human *Plasmodium* species, we recently showed that the malaria parasite reservoir in Central Vietnam was more complex than previously reported by standard microscopy [15]. We therefore added another secondary PCR, specific for *P. knowlesi*, into the existing SnM-PCR to enable the detection of the five *Plasmodium* species within a three-step PCR.

The existing SnM-PCR protocol was adapted from Rubio and others [14] and consists of a first reaction *Plasmodium* genus specific (Figure 1A) followed by a semi-nested PCR including the *Plasmodium*-specific forward (PLF) primer used in the primary PCR, plus species-specific reverse primers for *P. falciparum*, *P. vivax*, *P. malaria* and *P. ovale* (Figure 1B). We hereby describe the newly added semi-nested (Sn-) PCR for the identification of *P. knowlesi* (Figure 1C). The analysis of existing oligonucleotide primers for *P. knowlesi* showed that the reverse complement of the PkF1140 primer designed and validated by Imwong and others [6] was compatible with the PLF primer described by Rubio and others [14]. Therefore, the Sn-PCR of *P. knowlesi* includes the PLF forward primer, and a species-specific reverse primer and a species-specific reverse primer (PKR4: 3'-GAT TCA TCT ATT AAA AAT TTG CT-5'), the latter being the reverse complement of the PkF1140 primer shortened by two base pairs (bp). The specificity of the PKR4 was subsequently assessed *in silico* by aligning sequences for the 18S ssu rRNA genes of different human and non-human *Plasmodium* species (*P. knowlesi*, *P. coatney*, *P. cynomolgi*, *P. inui*, *P. fragile*, *P. reichenowi*, *P. lophurae*, *P. gallinacae*) as well as 29 *P. knowlesi* sequences available on GenBank (<http://www.ncbi.nlm.nih.gov/genbank/index.html>).

The Sn-PCR was performed in 25µl reaction mixture with final concentration for a 1x reaction of 1x Qiagen loading buffer (10x Qiagen buffer, Hilden, Germany), 100 µM of each dNTP (Eurogentec, Seraing, Belgium), 0.5 µM PLF, 0.2 µM PKR4, 1 Unit of Qiagen

HotstarTaq plus polymerase and 2 µl DNA (a 1/500 dilution of the primary PCR product was used as the template). Cycling conditions were as follows: a 5 min denaturation and activation of the Qiagen HotstarTaq plus polymerase step at 94°C, followed by 30 cycles of 20 sec at 51°C for annealing, followed by elongation at 72°C for 1min and a 30 sec denaturation at 94°C. The final cycle was followed by an extension time of 10 min at 72°C. The PCR products were detected by 2% agarose gel stained with ethidium bromide and visualized under UV light.

Reference blood samples from *P. knowlesi*-infected patients (n=13) were kindly provided by the IMR, Kuala Lumpur. Furthermore, 80 blood samples (filter paper) from malaria infected patients collected during a previous survey carried out in Vietnam [15] were used to further validate the specificity of the PLF-PKR4 primers. DNA extraction was done using the QIAamp DNA Micro Kit (Qiagen, Hilden Germany).

Primary and nested PCR products were cloned into plasmid vectors (pCR4 -TOPO vector) and transformed into Mach1™ T1^B*Escherichia coli* cells using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Thirty cut out bacterial colonies, 10 from two distinct *P. knowlesi* samples harboring the amplified primary PCR fragment, and 10 harboring the secondary PCR fragment (five colonies for each of the two *P. knowlesi* samples) were purified and sequenced. In addition, DNA extracts from the 13 *P. knowlesi* reference samples were subjected to the new Sn-PCR and the amplified PCR fragments of expected size (actual size amplicon = 498 bp), were of the agarose gel, purified, cloned and sequenced. The sequences obtained were analyzed with the BioEdit program (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) and compared with available sequences in GenBank. All sequences obtained from plasmids with primary and nested PCR inserts were confirmed as *P. knowlesi*.

The sensitivity and specificity of our Sn-PCR protocol was assessed using two previously published nested PCR protocols for the detection of *P. knowlesi*: one using Pmk8-Pmkr9 primers [1] (hereafter called PCR1) and the one using PkF1160-PkR1550 primers [6] (hereafter called PCR2). The relative sensitivity was determined by a 10-fold serial dilution of the *P. knowlesi* H strain (*Plasmodium knowlesi* Genomic DNA - *P. knowlesi* H strain, MRA-456G obtained at the MR4 [<http://www.mr4.org/>]) using DNA extract from human blood collected from healthy donors. The analytic sensitivity was determined using a 10-fold serial dilution of the Primary PCR product of PCR 1 and 2 (using primers rPLU1 and 5). This PCR product was first cloned, extracted, and

sequenced. The extracted plasmid DNA concentration was determined using three measurements of the Nanodrop (Thermo Scientific, Landmeer, The Netherlands). A 10-fold serial dilution of the plasmid DNA containing the *P. knowlesi* fragment was made to determine the sensitivity of the different PCRs. The PCR1 showed the highest in both experiments with an analytical sensitivity at dilutions as low as 1 fg/μl compared with the 100 fg/μl for PCR2 and our Sn-PCR. In both panels the *P. knowlesi* MR4 H strain as well as the plasmid dilution series, our Sn-PCR and the PCR2 showed the same sensitivity.

The specificity of the three protocols was tested using different genomic DNA controls from *Plasmodium* species originating from monkeys (1 *P. cynomolgy*, 1 *P. simium*, 1 *P. fragile*; reference strains from NIMPE, Hanoi), and humans (4 *P. vivax*, 2 *P. falciparum*, 1 *P. ovale* and 1 *P. malariae*; reference strains from ITM, Antwerp), 1 *P. knowlesi* (IMR, Kuala Lumpur) as well as genomic DNA (gDNA) of non-Plasmodium origin (1 *Leishmania donovani*, 1 *Schistosoma mansoni*, 1 *Trypanosoma cruzi*, 1 HIV provirus, 1 *Mycobacterium tuberculosis* and 1 *Mycobacterium ulcerans*). Our Sn-PCR and PCR2 showed no cross-reaction while PCR1 showed a false positive *P. knowlesi* with a 153 bp band for one of the four *P. vivax* controls.

The specificity was further tested with the 80 clinical samples, previously analyzed by SnM-PCR for the identification of the four human *Plasmodium* species [15]. Filter paper dried blood spots were then tested for the presence of *P. knowlesi* using the three nested PCR protocols (Table 1). Results showed that PCR1 identified six *P.*

knowlesi infections whereas our Sn-PCR and PCR2 did not detect any. Sequencing confirmed the absence of *P. knowlesi* in all six samples, and the cross-reactivity of PCR1 primers with human DNA (97% homology to region 6853624 bp to 6853772 bp on the Human chromosome 16 genomic contig GenBank accession number NW001838290.1). No evidence of contamination of the PCR products was found.

Further simplification of the protocol into a five- species SnM-PCR should be feasible as preliminary data have shown that the *P. knowlesi* –specific primer was compatible *in silico* with the other four human *Plasmodium*-specific primers. This requires additional optimization efforts, however if successful, with further enhance the value of the current protocol.

Our modified SnM-PCR, offers the advantage of being feasible and affordable for poor income settings where real time PCR is not available yet, while reliably detecting the five human *Plasmodium* species within a three-step assay.

Table 1. Specificity of the three nested protocols using 80 clinical samples from Vietnam

Mono- & mixed malaria infections [@]	Total tested n	Positive result for <i>P. knowlesi</i> by protocol:		
		Nested PCR1* (Pmk8-Pmkr9)	Nested PCR2** (PkF1160-PkR1150)	Sn- PCR (PLF-PKR4)
<i>P. falciparum</i> (<i>P. f</i>)	38	-	-	-
<i>P. vivax</i> (<i>P. v</i>)	14	-	-	-
<i>P. malariae</i> (<i>P. m</i>)	5	-	-	-
<i>P. ovale</i> (<i>P. o</i>)	1	-	-	-
<i>P.f</i> + <i>P.v</i>	7	2	-	-
<i>P.f</i> + <i>P.m</i>	3	-	-	-
<i>P.m</i> + <i>P.o</i>	1	-	-	-
<i>P.f</i> + <i>P.v</i> + <i>P.m</i>	5	2	-	-
<i>P.f</i> + <i>P.m</i> + <i>P.o</i>	2	1	-	-
<i>P.v</i> + <i>P.m</i> + <i>P.o</i>	3	1	-	-
<i>P.f</i> + <i>P.v</i> + <i>P.m</i> + <i>P.o</i>	1	-	-	-
Total (N)	80	6	0	0

@ Previously confirmed by SnM PCR [15].

*Following protocol by Singh and others [1].

** Following protocol by Imwong and others [6].

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Disclosure: The *P. knowlesi* H strain was obtained through the MR4 as part of the BEI Resources Repository, NIAID, NIH: *Plasmodium knowlesi* Genomic DNA- *P. knowlesi* H strain, MRA-456G, deposited by A. W. Thomas.

References

1. Singh B, Kim Sung L, Matusop A, Radhakrishnan A, Shamsul SS, Cox-Singh J, Thomas A, Conway DJ: A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. *Lancet* 2004, 363: 1017-1024.
2. Cox-Singh J, Singh B: *Knowlesi* malaria: newly emergent and of public health importance? *Trends Parasitology* 2008 24: 406-410.
3. Cox-Singh J, Davis TM, Lee KS, Shamsul SS, Matusop A, Ratnam S, Rahman HA, Conway DJ, Singh B: *Plasmodium knowlesi* malaria in humans is widely distributed and potentially life threatening. *Clin Infect Dis* 2008, 46: 165-171.
4. Vythilingam I, Noorazian YM, Huat TC, Jiram AI, Yusri YM, Azahari AH, Norparina I, Noorain A, Lokmanhakim S: *Plasmodium knowlesi* in humans, macaques and mosquitoes in peninsular Malaysia. *Parasites & Vectors* 2008, 1: 26.
5. White NJ: *Plasmodium knowlesi*: the fifth human malaria parasite. *Clin Infect Dis* 2008, 46: 172-173.
6. Imwong M, Tanomsing N, Pukarittayakemee S, Day NP, White NJ, Snounou G: Spurious amplification of *P. vivax* small subunit RNA gene using the primers currently used to detect *P. knowlesi*. *J Clin Microbiol* 2009, 47: 4173-4175.
7. Babady NE, Sloan LM, Rosenblatt JE, Pritt BS: Detection of *Plasmodium knowlesi* by Real-Time Polymerase Chain Reaction. *Am J Trop Med Hyg* 2009, 81: 516–518.
8. Iseki H, Kawai S, Takahashi N, Hirai M, Tanabe K, Yokoyama N, Igarashi I: Evaluation of a Loop-Mediated Isothermal Amplification (LAMP) method as a diagnostic tool of zoonotic simian malaria parasite *Plasmodium knowlesi* infection. *J Clin Microbiol* 2010, 48: 2509-2514.
9. Divis PCS, Shokoples SE, Singh B, Yanow SK: A TaqMan real-time PCR assay for the detection and quantitation of *Plasmodium knowlesi*. *Malar J* 2010, 9:344.
10. Lucchi NW, Poorak M, Oberstaller J, DeBarry J, Srinivasamoorthy G, Goldman I, Xayavong M, da Silva AJ, Peterson DS, Barnwell JW, Kissinger J, Udhayakumar V:

- A new single-step PCR assay for the detection of the zoonotic malaria parasite *Plasmodium knowlesi*. *PLoS ONE* 2012, 7: e31848.
11. Chew CH, Lim YAL, Lee PC, Mahmud R, and Chua KH: A hexaplex PCR detection system for the identification of five human *Plasmodium* species with internal control. *J Clin Microbiol* 2012, 50: 4012-4019.
 12. Van den Eede P, Nguyen Van H, Van Overmeir C, Vythilingam I, Ngo Duc T, Xuan Hung L, Nguyen Manh H, Anne J, D'Alessandro U, Erhart A: Human *Plasmodium knowlesi* infections in Southern Vietnam. *Malar J* 2009, 8:249.
 13. Ministry of Health, National malaria control and elimination programme. National Institute of Malariology, Parasitology, and Entomology: National strategy for malaria control and elimination for the period of 2012-2015. Hanoi, December 2011. Internal Report. 2012
 14. Rubio JM, Post RJ, van Leeuwen WM, Henry MC, Lindergard G, Hommel M: Alternative polymerase chain reaction method to identify *Plasmodium* species in human blood samples: the semi-nested multiplex malaria PCR (SnM-PCR). *Trans R Soc Trop Med and Hyg* 2002, 96: S199--S204.
 15. Hong Nguyen V, Van den Eede P, Van Overmeir C, Thang Ngo D, Hung Le X, D'Alessandro U, and Erhart A: The distribution of human *Plasmodium* species in central Vietnam is complex with marked age-dependent prevalence of symptomatic and patent infections. *Am J of Trop Med Hyg* 2012, 87: 989–995.

Chapter 4

Human *Plasmodium knowlesi* infections

in young children in central Vietnam

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Abstract

Background: Considering increasing reports on human infections by *Plasmodium knowlesi* in Southeast Asian countries, blood samples collected during two large cross-sectional malariometric surveys carried out in a forested area of central Vietnam in 2004 and 2005 were screened for this parasite.

Methods: Blood samples collected at the 2004 survey and positive for *Plasmodium malariae* were randomly selected for PCR analysis detecting *P. knowlesi*. Blood samples collected in 2005 from the same individuals were screened again for *P. knowlesi*. Positive samples were confirmed by sequencing. Family members of positive cases who participated in both surveys were also screened.

Results: Ninety-five samples with *P. malariae* mono- or mixed infections identified by species-specific PCR were screened for *P. knowlesi*. Among the five (5.2%) positive samples by PCR, three were confirmed to be *P. knowlesi* infections by sequencing, two young children (<5 years old) and a young man, all asymptomatic at the time of the survey and for the next six months after the survey. One of the two children was still positive one year later. No infection was found among the family members.

Conclusion: *Plasmodium knowlesi* infections in humans can be found in central Vietnam. A small child was positive for *P. knowlesi* in both surveys at one year interval, though it is unclear whether it was the same or a new infection.

Background

Plasmodium knowlesi has been recently defined as the “fifth human malaria species” [1] following the discovery in Malaysian Borneo of a large focus (58% of malaria cases in the Kapit hospital) of this simian malaria parasite in humans [2] and the more sporadic occurrence of other human cases in several Asian countries such as Thailand [3, 4], Myanmar [5], The Philippines [6], and Singapore [7]. *Plasmodium knowlesi*, though usually found in long-tailed and pig-tailed macaques (*Macaca fascicularis* and *Macaca nemestrina*) in Southeast Asian (SEA) forested areas, can be naturally transmitted to humans by vectors belonging to the *Anopheles leucosphyrus* group, e.g. *Anopheles latens* in Malaysian Borneo and *Anopheles cracens* in Peninsular Malaysia [2, 8, 9]. *Plasmodium knowlesi*, genetically closely related to *Plasmodium vivax* [2, 10], shares microscopically similarities with *Plasmodium malariae* and is characterized by a 24h erythrocytic cycle. It may cause severe illness with risk of fatal outcome and it occurs without obvious clustering of cases in human settlements [2, 11]. Known risk factors are adult age, forest-related activities or a recent travel history to forested areas [12 - 14]. Considering the increasing number of reports of human *P. knowlesi* infections in several SEA countries, blood samples collected during two large cross-sectional malariometric surveys carried out in 2004 and 2005 in a forested area in central Vietnam were screened for the presence of this parasite.

Materials and Methods

Study area

The field study aiming at evaluating the effectiveness of long-lasting insecticidal hammocks for controlling forest malaria was carried out between 2004 and 2006 in Ninh Thuan province, located in the southern part of central Vietnam, in a population of about 20,000 people [15, 16]. The study area is hilly and densely forested, inhabited mainly by the Ra Glai ethnic minority whose lifestyle is based on the exploitation of forest products, subsistence farming and cash crop cultivation. Beside their village homes, most families have plot huts in forest fields where they often stay overnight, particularly during the harvest season. Malaria transmission is perennial with two peaks (June and October) and is mainly supported by *Anopheles dirus sensu stricto* and *Anopheles minimus*, though several secondary vectors such as *Anopheles maculatus* and *Anopheles jeyporiensis* may be involved [17]. In 2004, the prevalence of malaria (all species) infection was 13.6% with a high proportion (>80%) of asymptomatic infections [16].

Detection of malaria clinical cases and infections

Malaria incidence and prevalence were estimated by combining cross-sectional surveys and passive case detection (PCD) at village level. For the latter, since July 2004, febrile patients attending either the Commune Health Centers (CHC) or consulting the village health workers (VHW) were identified, had the body temperature and a blood sample taken for immediate diagnosis (rapid diagnostic test, RDT) and later

microscopy. Patients were treated on the basis of the RDT results: *Plasmodium falciparum* (including mixed infections) with a seven-day course of artesunate (16mg/kg), and *P. vivax* with chloroquine (25mg/kg) for three days [16]. In addition, following the trial design, a cohort of more than 4,000 randomly selected individuals was surveyed bi-annually, before and after the rainy season (April & December) [15, 16]. At the time of the survey the body temperature was measured and the participants were interviewed about any symptoms in the previous 48 hours. Furthermore, blood smears for microscopy and blood spots were collected on filter paper (Whatman N°3 filter paper) for later molecular analysis (species-specific PCR).

Detection of *P. knowlesi* infections

Among the 210 *P. malariae* mono- or mixed infections identified by species-specific PCR on blood samples collected during the December 2004 survey, 95 were randomly selected to be screened for *P. knowlesi*. Forty-one of them were *P. malariae* mono-infections by species-specific PCR, while 54 were *P. malariae* mixed infections with either *P. falciparum* (15); *P. vivax* (15); *Plasmodium ovale* (5); *P. falciparum* and *P. vivax* (10); *P. vivax* and *P. ovale* (8); *P. falciparum*, *P. vivax* and *P. ovale* (1). By microscopy, 31 were negative, 42 mono-infections, *i.e.* 22 *P. falciparum*, 19 *P. vivax* and 1 *P. malariae*, and 11 mixed infections including two triple infections with *P. falciparum*, *P. vivax* and *P. malariae*. Family members of the *P. knowlesi* positive cases, included in the survey, were also screened for this parasite. Blood samples collected in December

2005 from the same individuals (index cases and their family members) were screened again for *P. knowlesi*.

Oral informed consent for blood sampling and malaria related analysis was obtained from all study participants after explanation of the study objectives and procedures in the local Ra Glai language.

PCR

The DNA was extracted from filter paper with the Saponine-chelex method [18]. A nested PCR assay described by Singh *et al* [2] that specifically amplifies one part of the *P. knowlesi* small subunit ribosomal RNA (SSUrRNA) gene was used to detect *P. knowlesi* DNA. Field samples confirmed (by species-specific PCR) to be mono-infections for *P. falciparum*, *P. ovale*, *P. malariae*, *P. vivax* [19], together with *P. knowlesi* positive samples from experimentally infected monkeys were used as controls. A negative control was included after 11 samples and at the end of each PCR. PCR conditions were as follows. The primary reaction was carried out in a 50 µl reaction mixture containing 1× reaction buffer (10x Qiagen Buffer), 3 mM MgCl₂ (Qiagen, Hilden Germany), 200 mM of each deoxynucleoside triphosphate (Eurogentec, Belgium), 250 nM of each primers (rPLU1: 5'-TCA AAG ATT AAG CCA TGC AAG TGA-3'; rPLU5: 5'-CCT GTT GTT GCC TTA AAC TCC3'), 0.1 µg/µl acetylated BSA (Promega, Madison USA) and 1 Unit HotStarTaq Plus DNA polymerase (Qiagen, Hilden Germany) and 2 µl of DNA template was used for each reaction. The nested PCR amplification was carried out in a 25 µl reaction mixture containing 1× reaction buffer

(10x Qiagen Buffer, Hilden Germany), 3 mM MgCl₂ (Qiagen, Hilden Germany), 200 mM of each deoxynucleoside triphosphate (Eurogentec, Belgium), 250 nM of each primers (Pmk8: 5'-GTT AGC GAG AGC CAC AAA AAA GCG AAT-3'; Pmk9r: 5'-ACT CAA AGT AAC AAA ATC TTC CGT A-3') and 2 Units HotStarTaq Plus DNA polymerase (Qiagen, Hilden Germany) and 2 µl of the primary PCR products were used as DNA templates. All PCR reactions were carried out using PTC 100 thermal cycler (Bio-Rad, California USA). 5 µl of the nested PCR products were loaded on a 2% agarose gel for 60 min at 5 V/cm using 0.5X TAE buffer. The gels were stained with ethidium bromide and visualized with UV.

Cloning and sequence analysis

Before cloning, the PCR was repeated for the *P. knowlesi* positive samples to confirm the results. The nested PCR products of *P. knowlesi* samples were cloned using TOPO cloning kit, (Invitrogen, California USA). The PCR products were inserted in the pCR4[®]-TOPO plasmid and grown in *E. coli* (Mach1-T1R cells). Once cloned, they were purified using the QuickLyse™ kit (Qiagen, Hilden Germany).

The cloned products were sequenced, and sequence alignment (with ClustalW [20]) was done with sequences of the 18S ribosomal sub-unit of *P. knowlesi* expressed during the sexual stages of the lifecycle or S-Type SSUrRNA gene, which were available in GenBank [GenBank accession numbers: DQ350263; DQ350262; DQ350261; DQ350260; DQ350259; DQ350258; DQ350257; DQ350256; DQ350255; DQ350271 DQ350270 and U83876].

Results

Ninety-five samples from the December 2004 survey were screened for *P. knowlesi*. Among the five (5.2%) initially found positive, only three remained positive after repeating the PCR. These were then confirmed by sequencing. One of the three index cases was re-confirmed to be positive one year later (December 2005 survey) for *P. knowlesi* (PCR and sequencing). The sample collected from this individual in 2004 had two sequences. The sequences (153 base pairs in size) obtained from the Vietnamese samples [GenBank accession numbers: FJ160750 FJ160751; FJ160752; FJ160753 and FJ871986] showed 97 - 99% similarity with the Malaysian strains.

Plasmodium knowlesi infections were found in two young children (two-year old boy and three-year old girl) and a 27-year old man, all asymptomatic at the time of the survey. The microscopy examination had identified a *P. falciparum* and *P. vivax* mixed infection (3,120 parasites/ μ l) in the three-year old girl and a *P. vivax* mono-infection (32 parasites/ μ l) in the young man; the blood slide was negative in the other child. By species-specific PCR, all three individuals carried a mixed infection, the three-year old girl had *P. falciparum*, *P. vivax* and *P. malariae*, the two-year old boy had *P. malariae* and *P. ovale* and the young man *P. vivax* and *P. malariae*.

Six months prior to the survey, the passive case detection had identified a probable clinical attack (*P. falciparum* detected by RDT) in the adult man. No other malaria clinical attack among the *P. knowlesi* positive cases was identified by passive case detection during the entire study period (July 2004 to December 2006).

In the December 2004 survey, five out of the nine family members tested by PCR were positive for *P. knowlesi* infection, even after repeating such analysis. However, none of them was confirmed by sequencing. In the 2005 survey, the three-year old girl was again found positive by PCR for *P. knowlesi* and confirmed by sequencing, while the two other index cases and all concerned family members, were positive by PCR but were not confirmed by sequencing. The latter results were due to aspecific reaction with human DNA, as the 161 base pairs fragment obtained by PCR displayed 97% similarity with a region of similar size of the human chromosome 16. The excess of human DNA and the possible binding sites for rPLU1 and rPLU5 were probably responsible for such aspecific reactions: rPLU1 and rPLU5 had respectively 80% and 76% similarity with their aspecific binding regions in the human genome.

All three confirmed *P. knowlesi* positive cases and their family members had a history of regular forest activities (children accompanying the adults), including nights frequently spent in the forest.

Discussion

This is the first report of human *P. knowlesi* infections in Vietnam, not an unexpected finding considering the numerous reports from neighboring countries [2 - 7]. Nevertheless, most previously reported cases were adults, often symptomatic, some of them with relatively high parasite densities. Instead, in this study, all *P. knowlesi* infections were asymptomatic, co-infected with *P. malariae*, with low parasite

densities and two of the three identified cases were very young children under five years of age.

The *P. knowlesi* infections reported here were detected retrospectively by analyzing blood spots on filter paper, thus the three individuals could not be followed up after the survey. Nevertheless, clinical malaria was carefully monitored in this population by passive case detection during which fever episodes were identified and checked for malaria infection. Though an undetected fever episode in the *P. knowlesi* infected individuals cannot be excluded with absolute certainty, none of the three *P. knowlesi* cases had any reported fever nor malaria-related consultation in the PCD during the whole study period (except the young adult man who was identified and treated for *P. falciparum* six months prior the 2004 survey). Moreover, during each of the subsequent surveys, none of the three patients had or reported fever.

One of the children still harbored the *P. knowlesi* infection one year later, though it is impossible to determine whether this was the same or a new infection. A possible approach would be the genotyping and comparison of both infections, though this would be helpful only if the parasite population was diverse enough. Finding an infection at one year interval in a young child indicates that in this area human exposure to *P. knowlesi* infection is not a rare event, either because the child may have been re-infected or because she was able to control the infection without developing symptoms, indicating some suppressing immunity.

The close contact of this community with the forest, where the *P. knowlesi* natural hosts can be found (monkeys are often kept as pets), is an additional element indicating a high risk of exposure to the infection. Indeed, the occurrence of *P. knowlesi* in SEA follows the distribution of its natural host, the long and pig tailed macaque [21], which mainly lives in the deep forest. Unlike other SEA countries, where recent economical development and deforestation have led to an increased invasion of the monkeys' habitat by man, in this Ra Glai community [22], the continuous contact with the forest probably means also a relatively constant exposure to *P. knowlesi* infection since the early years of life. Such exposure is made possible by the presence of an efficient vector that feeds both on humans and monkeys. The main malaria vector in central Vietnam, *An. dirus s.s.*, belongs to the *An. leucosphyrus* group, which also includes the two confirmed vectors of *P. knowlesi* in Malaysia, *An. latens* and *An. cracens* [9, 23, 24]. *Anopheles dirus s.s.* is a sylvatic species, usually highly anthropophilic which, in the absence of humans, can feed on monkeys especially in the canopy, and sporadically on other animals such as dogs, birds, and cattle [23]. Recently, *P. knowlesi* has been identified in an *An. dirus* specimen collected in central Vietnam (Khan Hoa province, neighboring the study area), indicating that *P. knowlesi* may be transmitted by this primary human malaria vector [25]. *Anopheles dirus s.s.* ability to transmit *P. knowlesi* from monkey to monkey under lab conditions has already been reported [26], though the natural transmission from monkey to man has not been confirmed.

Confirmation of the *P. knowlesi* infection by sequencing was needed as the *P. knowlesi* PCR assay in this study had a low specificity, resulting in a substantial proportion of false positives. This is clearly illustrated by the high proportion of PCR positive results among the family members of the index cases later identified to be aspecific amplifications of human DNA. This also suggests that further improvement and standardization of the PCR technique is needed. Until there is no reliable and simple diagnostic test for detecting *P. knowlesi* infection, appreciating its distribution and burden in human populations will remain extremely difficult.

Conclusion

Plasmodium knowlesi infection is present in Vietnam and has been found in a Ra Glai community living in a relatively remote area in close contact with the forest. Two of the three identified infections occurred in young children below five, and in one of them, *P. knowlesi* was identified again one year later, indicating that human exposure to *P. knowlesi* infection is not a rare event. Interestingly, none of the *P. knowlesi* positive cases had malaria clinical symptoms either during the surveys, or during passive case detection until one year after each survey.

A better estimation of the importance of *P. knowlesi* infection in this population, as well as in other forested areas in central Vietnam would need larger studies. However, considering the difficulties in diagnosing *P. knowlesi* infections, simpler and more specific diagnostic tools are urgently needed.

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References

1. White NJ: *Plasmodium knowlesi*: the fifth human malaria parasite. *Clin Infect Dis* 2008, *46*: 172-173.
2. Singh B, Kim Sung L, Matusop A, Radhakrishnan A, Shamsul SS, Cox-Singh J, Thomas A, Conway DJ: A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. *Lancet* 2004, *363*: 1017-1024.
3. Jongwutiwes S, Putaporntip C, Iwasaki T, Sata T, Kanbara H: Naturally acquired *Plasmodium knowlesi* malaria in human, Thailand. *Emerg Infect Dis* 2004, *10*: 2211-2213.
4. Putaporntip C, Hongsrimuang T, Seethamchai S, Kobasa T, Limkittikul K, Cui L, Jongwutiwes S: Differential prevalence of Plasmodium infections and cryptic *Plasmodium knowlesi* Malaria in Humans in Thailand. *J Infect Dis* 2009, *199*: 1143-1150.
5. Zhu HM, Li J, Zheng H: Human natural infection of *Plasmodium knowlesi*. *Chinese Journal of Parasitology and Parasitic diseases* 2006, *24*: 70-71.
6. Luchavez J, Espino F, Curameng P, Espina R, Bell D, Chiodini P, Nolder D, Sutherland C, Lee KS, Singh B: Human Infections with *Plasmodium knowlesi*, the Philippines. *Emerg Infect Dis* 2008, *14*(5): 811-813.
7. Ng OT, Ooi EE, Lee CC, Lee PJ, Ng LC, Pei SW, Tu TM, Loh JP, Leo YS: Naturally acquired human *Plasmodium knowlesi* infection, Singapore. *Emerg Infect Dis* 2008, *14*: 814-816.
8. Vythilingam I, Noorazian YM, Huat TC, Jiram AI, Yusri YM, Azahari AH, Norparina I, Noorain A, Lokmanhakim S: *Plasmodium knowlesi* in humans, macaques and mosquitoes in peninsular Malaysia. *Parasit Vectors* 2008, *1*: 26.
9. Vythilingam I, Tan CH, Asmad M, Chan ST, Lee KS, Singh B: Natural transmission of *Plasmodium knowlesi* to humans by *Anopheles latens* in Sarawak, Malaysia. *Trans R Soc Trop Med Hyg* 2006, *100*: 1087-1088.

10. Carlton JM, Adams JH, Silva JC, Bidwell SL, Lorenzi H, Caler E, Crabtree J, Angiuoli SV, Merino EF, Amedeo P, Cheng Q, Coulson RM, Crabb BS, Del Portillo HA, Essien K, Feldblyum TV, Fernandez-Becerra C, Gilson PR, Gueye AH, Guo X, Kang'a S, Kooij TW, Korsinczky M, Meyer EV, Nene V, Paulsen I, White O, Ralph SA, Ren Q, Sargeant TJ, Salzberg SL, Stoeckert CJ, Sullivan SA, Yamamoto MM, Hoffman SL, Wortman JR, Gardner MJ, Galinski MR, Barnwell JW, Fraser-Liggett CM: Comparative genomics of the neglected human malaria parasite *Plasmodium vivax*. *Nature* 2008, *455*: 757-763.
11. Cox-Singh J, Davis TM, Lee KS, Shamsul SS, Matusop A, Ratnam S, Rahman HA, Conway DJ, Singh B: *Plasmodium knowlesi* malaria in humans is widely distributed and potentially life threatening. *Clin Infect Dis* 2008, *46*: 165-171.
12. Bronner U, Divis PC, Färnert A, Singh B: Swedish traveller with *Plasmodium knowlesi* malaria after visiting Malaysian Borneo. *Malar J* 2009, *8*: 15.
13. Kantele A, Marti H, Felger I, Müller D, Jokiranta TS: Monkey malaria in a European traveler returning from Malaysia. *Emerg Infect Dis* 2008, *14*: 1434-1436.
14. Cox-Singh J, Singh B: Knowlesi malaria. Newly emergent and of public health importance? *Trends Parasitol* 2008, *24*: 406-410.
15. Thang ND, Erhart A, Speybroeck N, Xa NX, Thanh NN, Ky PV, Hung le X, Thuan le K, Coosemans M, D'Alessandro U: Long-lasting insecticidal hammocks for controlling forest malaria: a community-based trial in a rural area of central Vietnam. *PLoS One* 2009, *4*: e7369.
16. Thang ND, Erhart A, Hung le X, Thuan le K, Xa NX, Thanh NN, Ky PV, Coosemans M, Speybroeck N, D'Alessandro U: Rapid decrease of malaria morbidity following the introduction of community-based monitoring in a rural area of central Vietnam. *Malar J* 2009, *8*: 3.
17. Trung HD, Bortel WV, Sochantha T, Keokenchanh K, Briët OJ, Coosemans M: Behavioural heterogeneity of Anopheles species in ecologically different

- localities in Southeast Asia: a challenge for vector control. *Trop Med Int Health* 2005, 10: 251-262.
18. Plowe CV, Djimde A, Bouare M, Doumbo O, Wellems TE: Pyrimethamine and proguanil resistance-conferring mutations in *Plasmodium falciparum* dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa. *Am J Trop Med Hyg* 1995, 52: 565-568.
 19. Rubio JM, Post RJ, van Leeuwen WM, Henry MC, Lindergard G, Hommel M: Alternative polymerase chain reaction method to identify Plasmodium species in human blood samples: the semi-nested multiplex malaria PCR (SnM-PCR). *Trans R Soc Trop Med Hyg* 2002, 96 (Suppl 1): S199-S204.
 20. EMBL-EBI website sequence analysis clustalW2, [<http://www.ebi.ac.uk/Tools/clustalw2/index.html>], accessed 28 September 2009.
 21. Primate Info Net [<http://pin.primate.wisc.edu/factsheets>], accessed 20 March 2009.
 22. Dang Nghiem Van, Chu Thai Son, Luu Hung: Ethnic minorities in Vietnam. The Gioi Publisher; 2000. Hanoi.
 23. Obsomer V, Defourny P, Coosemans M: The *Anopheles dirus* complex: spatial distribution and environmental drivers. *Malar J* 2007, 6: 26.
 24. Tan CH, Vythilingam I, Matusop A, Chan ST, Singh B: Bionomics of *Anopheles latens* in Kapit, Sarawak, Malaysian Borneo in relation to the transmission of zoonotic simian malaria parasite *Plasmodium knowlesi*. *Malar J* 2008, 7: 52.
 25. Nakazawa S, Marchand RP, Quang NT, Culleton R, Manh ND, Maeno Y: *Anopheles dirus* co-infection with human and monkey malaria parasites in Vietnam. *Int J Parasitol* 2009, 39(14): 1533-1537.
 26. Collins WE, Contacos PG, Guinn EG: Studies on the transmission of simian malarias II. Transmission of the H strain of *Plasmodium knowlesi* by *Anopheles balabacensis* balabacensis. *J Parasitology* 1967, 53: 841-844.

Chapter 5

Marked age – dependent prevalence of symptomatic and patent infection and complexity of distribution of human *Plasmodium* species in central Vietnam

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Abstract

In Vietnam, *Plasmodium falciparum* and *Plasmodium vivax* are responsible for most malaria infections, and *Plasmodium malariae* and *Plasmodium ovale* infections are rarely reported. Nevertheless, species specific polymerase chain reaction analysis on 2,303 blood samples, collected during a cross sectional survey conducted in a forested area of central Vietnam, identified 223 (9.7%) *P. falciparum*, 170 (7.4%) *P. vivax*, 95 (4.1%) *P. malariae*, 19 (0.8%) *P. ovale* mono-infections and 164 (7.1%) mixed infections. Out of the 671 *Plasmodium*-positive samples by polymerase chain reaction, only 331 were detected by microscopy. Microscopy poorly diagnosed *P. malariae*, *P. ovale* and mixed infections. Clinical and sub-clinical infections occurred in all age groups. The risk for infection and disease decreased with age, probably because of acquired partial immunity. The common occurrence of sub-patent infections seems to indicate that the malaria burden is underestimated and that diagnostic and therapeutic policies should be adapted accordingly.

Introduction

Since the 1990s the malaria burden in Vietnam has been efficiently reduced (> 90%) by the national malaria control program and malaria now confined to the remote and forested areas populated mainly by poor ethnic minorities in which a large proportions ($\leq 80\%$) of asymptomatic and untreated infections with low parasite densities can be found [1,2]. *Plasmodium falciparum* and *Plasmodium vivax* are the most commonly identified malaria species, with a few rare reports of *Plasmodium malariae* and *Plasmodium ovale* infections [1, 3, 4]. Nevertheless, routine detection and management of malaria is based on microscopy (thick blood film) that has a limited sensitivity (± 50 parasites/ μl blood). Consequently, low density infections and mixed infections may be missed. Sub-patent malaria infections, detectable only by molecular techniques (polymerase chain reaction [PCR]), can be relatively common in areas of low malaria endemicity in Southeast Asia and South America and may substantially contribute in maintaining malaria transmission in these areas [5-10].

Because Vietnam has reached a pre-elimination stage, with malaria having been eliminated in most of its territory, further successes and ultimately elimination, require more sensitive diagnostic tools that are able to successfully detect residual foci of low transmission for better targeting control/elimination efforts. However, because PCR-based diagnosis is limited by its cost, the need of specific equipment and trained personnel, malaria cases reported by the national health information system are almost exclusively based on microscopy identification. To assess more precisely the

burden and complexity of human malaria infections in central Vietnam, we retrospectively analyzed by PCR blood samples collected in a large cross-sectional survey conducted within the framework of a project evaluating the effectiveness of insecticide-treated hammocks.

Materials and methods

Sample collection

Filter paper blood samples collected during a large-scale malariometric survey conducted in November - December 2004, in a rural area of central Vietnam (Ninh Thuan province) were retrospectively selected to be analyzed by species-specific PCR. Details on the survey methods and study site have been reported [1]. In brief, the study population was composed mainly of persons of the Ra-glai ethnic minority, whose subsistence is based on forest farming and products exploitation [11]. Malaria transmission is low and perennial in the study region, with two annual peaks (at the beginning and end of the rainy season), and is mainly caused by *Anopheles dirus sensu stricto*, a sylvatic species and an efficient malaria vector with exophagic and exophillic behavior.

The survey was conducted in the framework of a community-based cluster randomized trial on the effectiveness of long-lasting insecticide-treated hammocks in 20 clusters of approximately 1,000 habitants each. The survey, which was part of the baseline study and was conducted just before implementation of the intervention, involved 4,090 persons randomly selected from the study population. After interview

and clinical examination, survey participants provided a finger prick blood sample for microscopy (thick and thin blood films) and for molecular and serologic tests. Blood samples were placed on Whatman grade 3 filter paper (Whatman, Springfield Mill, United Kingdom). After drying, filter paper blood samples were kept individually in plastic bags with silica gel and stored at room temperature in a dark and dry place.

Protocols and results of microscopy examination of blood smears have been reported [1]. Slide reading and quality control (double reading) was performed by senior technicians at the National Institute for Malaria, Parasitology and Entomology (Hanoi, Vietnam). Unfortunately, for the present study, blood smears were not available for external quality control (post-PCR). Therefore microscopy results are those reported originally.

Sample size

The previously determined parasite rate by microscopy in the survey was estimated to be 17.7% with a precision of 4.5% [1]. Thus, taking the lower limit of the confidence interval (CI) (13%) as a minimum prevalence by cluster, a minimum of 100 filter paper blood samples per cluster (plus a safety margin of 10%) would enable detecting a minimum parasite rate by PCR of 13% with a 6% precision at a 95% confidence level. A total of 2,303 filter paper blood samples were analyzed by species-specific PCR.

Species-specific semi nested multiplex PCR

DNA was extracted from filter paper by using the saponine-chelex method, and the PCR was performed according to a described method with primers specific for the 18S rDNA region [12, 13]. The PCR products were subjected to electrophoresis on a 2% agarose gel for 60 minutes at 5V/cm with 0.5x Tris-acetate EDTA buffer. The gels were stained with ethidium bromide, and visualized with ultraviolet light. The sizes of the PCR products were compared with a standard 100-basepair DNA ladder (Fermentas, Burlington Ontario, Canada) and positive controls of each *Plasmodium* species. Precautions for cross- contamination during handling were taken by implementing negative controls in each step from extraction to the nested PCR step. Approximately 5% (n = 120) of the samples were repeated blindly and results were confirmed by a senior technician.

The following case definitions were used. Sub-patent malaria infections were defined as malaria infections detected by PCR but negative by microscopy (absence of trophozoites and gametocytes after examining 1,000 leukocytes. Asymptomatic malaria infections were defined as PCR-detected malaria infections (regardless of microscopy results) without either fever at the time of sampling (body temperature < 37.5°C) or history of fever during the 3 days before sampling. Symptomatic malaria infections were defined as PCR- detected malaria infection (regardless of microscopic results) with fever at the time of sampling and/or history fever during 3 days before sampling). A microscopically positive slide was defined as any slide with either asexual

or sexual *Plasmodium* stage, or both. For the purpose of our study, inclusion of gametocytes among the positive slides was justified by the need of having the microscopy comparable to PCR because PCR detects asexual and sexual stages. Parasite density was defined as the density of the predominant species in each infection. Gametocyte prevalence was defined as the prevalence of malaria infection (detected by PCR) carrying gametocytes (identified by microscopy) of any of the four species.

Data analysis

Data were entered in Excel (Microsoft, Redmond, WA) and analyzed by using Stata version 10 software (Stata Corp LP, College Station, TX). Descriptive statistics were used to compute malariometric indices and a survey χ^2 test (svytab command in STATA) was used to test for significant differences in proportions ($P < 0.05$). The prevalence of all malaria infections (all PCR-detected infections), patent/sub-patent and symptomatic/asymptomatic infections and gametocytes carriage were computed by age group. The effect of age on the risk of malaria infection was adjusted for previously defined confounders such as forest work, bed net use and socioeconomic status in a multivariate survey logistic regression (svylogit command in STATA) to take into account the cluster design [14]. Subsequently, among all malaria infected cases, the risk of patent infection (compared with sub-patent infection), the risk of symptomatic infection (compared with asymptomatic infection), the risk of infections with gametocytes (compared with those without gametocytes), and the risk of mixed

infections (compared with mono-infections) were similarly examined by age groups and adjusted for the above mentioned confounders in four survey logistic regression models.

Ethical considerations

The protocol of the cluster randomized trial was approved by the Institutional Review Board of the Institute of Tropical Medicine and by the Ethical Committee of the University Hospital (both in Antwerp, Belgium). In Vietnam, the protocol was approved by the National Institute of Malariology, Parasitology and Entomology (Hanoi) and by the Ministry of Health [1].

Results

Malaria species distribution and prevalence

Among the 2,303 filter paper blood samples analyzed, 671 were positive by PCR, resulting in an overall parasite prevalence of 29.1% (95% CI =23.3-35.8. Most of patients had a *Plasmodium falciparum* mono-infection (prevalence = 9.7%) followed by *P. vivax* (7.4%), *P. malariae* (4.1%) and *P. ovale* (0.8%) mono-infections (Table 1). Approximately 25% of all infections yielded more than one *Plasmodium* species (prevalence of mixed infections = 7.1%); ≤ 3 species could be identified in 16% of the mixed infections. Co-infections with *P. malariae* were the most common (66.5%), followed by those including *P. vivax* or *P. falciparum*. Interestingly, *P. ovale* was found in 30.5% (50 of 164) of all mixed infections. The overall prevalence of *P. ovale* was high

(3%, 69 of 2,303) and this species was mostly observed in mixed infections (72%, 50 of 69 of the cases). This trend was shared by *P. malariae*, which also occurred in 53% of the cases (109 of 204) in co-infections, while *P. falciparum* and *P. vivax* preferentially occurred as mono-infections (70%, 223 of 319 and 63%, 170 of 269 respectively).

Among the 671 samples positive by PCR, only in 331 (49%) individuals the malaria infection was also detected by microscopy, suggesting that sub-patent malaria infections (14.8%, 340 of 2,303) were at least as prevalent as patent infections (14.4%) in this population. The microscopically determined species distribution showed a majority of *P. falciparum* mono-infections (n = 149) followed by *P. vivax* (n = 125); only two *P. malariae* and no *P. ovale* infections were identified. The prevalence of microscopically detected mixed infections was only 2.4% (n = 55), all of them *P. vivax* co-infections, mostly with *P. falciparum* (n = 50).

Discrepancies between results of microscopy and PCR were common. However, because blood smears were not available for an independent external re-examination, individual comparisons between microscopy and molecular results would be spurious. Therefore, the main characteristics of discrepant results were analyzed after grouping PCR results by mono- or mixed infections. Among the PCR-

identified *P. malariae* and *P. ovale* mono-infections (n = 114), only 28% (n = 32) were detected by microscopy but most of them were identified as *P. falciparum* or *P. vivax* mono-infections with a median parasite density of 56 parasites/ μ l (interquartile range [IQR]: 12 -320 parasites/ μ l). Among the PCR-identified *P. falciparum* and *P. vivax*

mono-infections, 48% (187 of 393) were detected by microscopy and most (87%, 144 of 187) were identified as *P. falciparum* and *P. vivax* mono-infections, with a median parasite density at 160 parasites/ μ l (IQR = 40-960 parasites/ μ l).

Of the 164 mixed infections, 68% (112 of 164) were positive by microscopy but only 23% (25 of 112) were identified as mixed infections; the remaining were classified as either *P. falciparum* or *P. vivax* mono-infections. The median parasite density in mixed infections correctly identified as such by microscopy and PCR was 240/ μ l (IQR =144 - 760 parasites/ μ l). Among those classified as either *P. falciparum* or *P. vivax* mono-infections, the median parasite density was lower (68 parasites/ μ l, IQR = 24 -480 parasites/ μ l). Among PCR negative samples, 82 (5%) were positive by microscopy, most of them either *P. falciparum* or *P. vivax* mono-infections with a low parasite density (median density = 32 parasites/ μ l, IQR =16-72 parasites/ μ l). Because slides results could not be cross-checked, for the purpose of the present analysis these infections were considered as false positives results, although the presence of an infection cannot be excluded with certainty because PCR analysis may have failed because of the presence of inhibitors, sample degradation, mutations at primer sites and errors during handling.

Table 1. Malariometric indices based on PCR and microscopy detection, central Vietnam*

Malaria indices, (n = 2,303)	PCR. no (%)	Microscopy⁺, no(%)
Overall malaria prevalence	671 (29.1)	331 (14.4)
Mono-infection: mixed infections (%)	507:164 (3.1)	276:55 (5.0)
Malaria prevalence by species		
<i>P.falciparum</i>	223 (9.7)	149(6.5)
<i>P.vivax</i>	170 (7.4)	125 (5.4)
<i>P.malariae</i>	95 (4.1)	2 (0.1)
<i>P.ovale</i>	19 (0.8)	0
Mixed	164 (7.1)	55 (2.4)
<i>P. malariae</i>	109 (66.5)	5 (9.10)
<i>P. vivax</i>	99 (60.4)	55 (100.0)
<i>P. falciparum</i>	96 (58.5)	50 (90.9)
<i>P. ovale</i>	50 (30.5)	0
Prevalence of gametocytes ⁺⁺	-	144 (6.3)
Prevalence of sub-patent infections	340 (14.7)	-
Prevalence of asymptomatic infections	547 (23.8)	265 (11.5)

*PCR = polymerase chain reaction.

⁺Microscopy examination results of the 671 PCR- positive samples (all PCR – negative samples were considered negative for microscopy for the purpose of this report)

⁺⁺Microscopically detected gametocytes.

Age-dependent risk for malaria infection and symptoms

The prevalence of all PCR-detected malaria infections decreased from 32.5% in the group less than five years of age to 26.2% in adults \geq 30 years of age, with a slow but

significantly decreasing trend ($P < 0.005$, by score test for trend) (Figure 1). Interestingly, the prevalence of patent infections across age groups was reduced by more than 60% (from 22.5% to 9.4%) and the prevalence of sub-patent infections increased by 69% (from 10% to 16.9%). This finding illustrated by the evolution of sub-patent: patent infections ratio, which was 0.4 in the persons less than five years of age, approximately 0.8 in person's ≤ 19 years of aged, and approximately 2 in adults.

Multivariate adjusted analysis for the risk of malaria infection (all PCR positive) (Table 2) by age group showed that the decreasing trend in the likelihood of malaria infection became significant from the age of 20 years with an odds of infections that was 35% lower in adults compared with children less than five years of age (adjusted odds ratio (AOR) = 0.65; 95% CI = 0.46- 0.92). Similarly, compared with sub-patent infections, the likelihood of patent infections (Table 2) decreased with age though not significantly until the age of 19 years (AOR = 0.38, 95% CI =0.12- 1.18), but significantly from in those ≥ 20 years of age; adults were > 80% less at risk compared with persons less than five years of age (AOR = 0.18, 95% CI =0.06- 0.43).

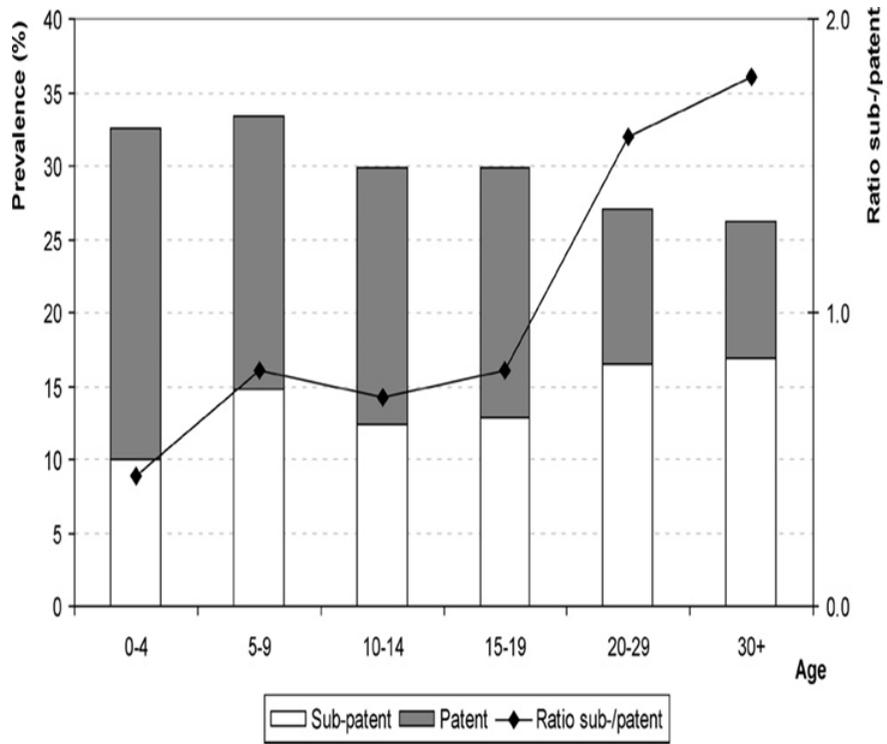


Figure 1. Prevalence of patent (grey bars) and sub-patent (white bars) malaria infections by age group, central Vietnam

Table 2. Multivariate adjusted analysis for the risk of malaria infection by age group (survey logistic regression models), central Vietnam

Characteristic		No. positive/ no. tested	AOR	P	95% CI
All malaria infections*					
Age groups, years	0-4	68/209	1		
	5-9	142/425	1.17	0.26	0.88 - 1.54
	10-14	94/315	0.89	0.53	0.59 - 1.32
	15-19	70/235	0.77	0.27	0.47 - 1.26
	20-29	126/467	0.65	0.02	0.46 - 0.92
	≥30	171/652	0.65	0.03	0.45 - 0.96
Patent infections**					
Age groups, years	0-4	47/68	1		
	5-9	79/142	0.52	0.13	0.22 - 1.24
	10-14	55/94	0.5	0.08	0.23 - 1.09
	15-19	40/70	0.38	0.09	0.12 - 1.18
	20-29	49/126	0.18	0.001	0.08 - 0.44
	≥30	61/171	0.16	0.001	0.06 - 0.43
Gametocyte carriage**°					
Age groups, years	0-4	29/68	1		
	5-9	40/142	0.49	0.03	0.26 - 0.93
	10-14	22/94	0.31	0.00	0.16 - 0.63
	15-19	15/70	0.25	0.01	0.1 - 0.70
	20-29	18/128	0.15	0.00	0.06 - 0.35
	≥30	20/171	0.12	0.00	0.06 - 0.24
Mixed infections**°					
Age groups, years	0-4	21/68	1		
	5-9	38/142	0.8	0.49	0.42 - 1.55
	10-14	22/94	0.48	0.09	0.21 - 1.12
	15-19	15/70	0.41	0.01	0.21 - 0.79
	20-29	34/126	0.53	0.1	0.25 - 1.13
	≥30	34/171	0.34	0.02	0.14 - 0.80
Symptomatic infection**°					
Age groups, years	0-4	19/68	1		
	5-9	25/142	0.58	0.196	0.245 - 1.362
	10-14	19/94	0.54	0.088	0.266 - 1.105
	15-19	15/70	0.51	0.119	0.210 - 1.214
	20-29	23/126	0.37	0.015	0.171 - 0.809
	≥30	23/171	0.26	0.003	0.111 - 0.594

AOR= adjusted odd ratio; CI= confidence interval

*Adjusted for forest work, socio-economic level, and bed net use.

°Among all infected individuals

The marked decrease in age-specific prevalence of patent infections correlated with a similar decrease (77%) in the prevalence of gametocytes from 13.9% to 3.1% (Figure 2). In the multivariate adjusted model (Table 2), the odds of carrying gametocytes among infected persons was also > 80% lower in the adult groups (≥ 20 years of age) compared with children less than five years of age. Interestingly, the prevalence of mixed infections also decreased with age (from 10.1% to 5.2%), and the prevalence of mono-infections did not decrease. In the multivariate adjusted model (Table 2) infected adults (> 30 years old) were 66% less at risk of having a mixed infection (compared with a mono-infection) compared with infected children less than five year of age. The age-related decrease of all microscopically defined indices (patent, mixed species and gametocyte carrying infections) correlated well with a significant reduction (more than 10-fold) of the mean parasite density (geometric mean), which decrease from 883 parasites/ μl (95% CI = 518- 1,519) in persons less than five years of age to 61 parasites/ μl (95% CI = 42- 88) in adults ≥ 30 years of age (Figure 2).

Most (81.5%) malaria infections detected by PCR were asymptomatic at the time of the survey, and the proportion of asymptomatic infections was similar between sub-patient and patient infections, i.e., 17.1% (58 of 340) and 19.9% (66 of 331), respectively. The prevalence of symptomatic infections decreased progressively, from 9.1% persons less than five years of age to 3.5% in adults and the prevalence of asymptomatic infections remained stable at approximately 23% (Figure 3). The evolution of symptomatic infections with age mirrored the evolution of sub-patient

infections. There was an overall decreasing trend with age in the proportion of symptomatic infections, i.e., from 28% in children less than five years of age to 12% in adults ≥ 30 years of age, similarly in sub-patent and patent infections.

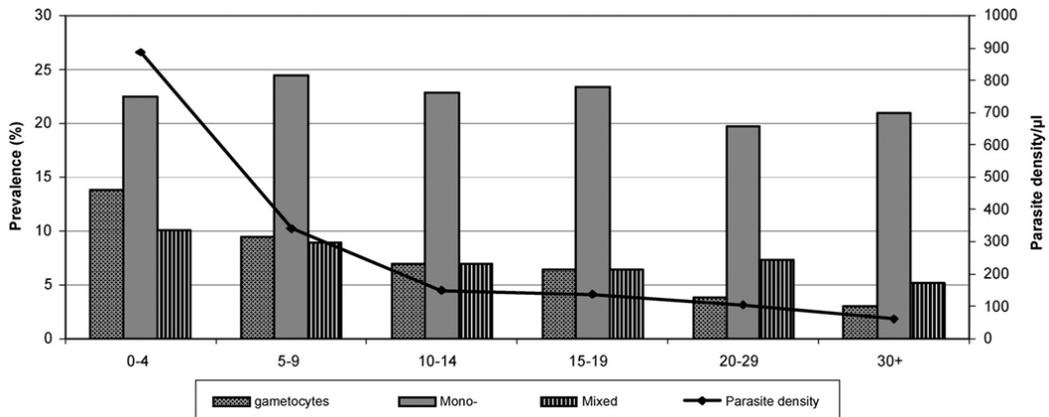


Figure 2. Prevalence of gametocyte carriage and mixed infections together with mean parasite density (geometric mean) by age group, central Vietnam

Mono= mono-infection.

The multivariate adjusted risk of symptomatic infection among infected individuals (Table 2) decreased by 63% in young adults (20-29 years of age, AOR = 0.37, 95% CI =0.17- 0.81) and by almost 75% in adults (≥ 30 years of age) compared with children less than five years of age.

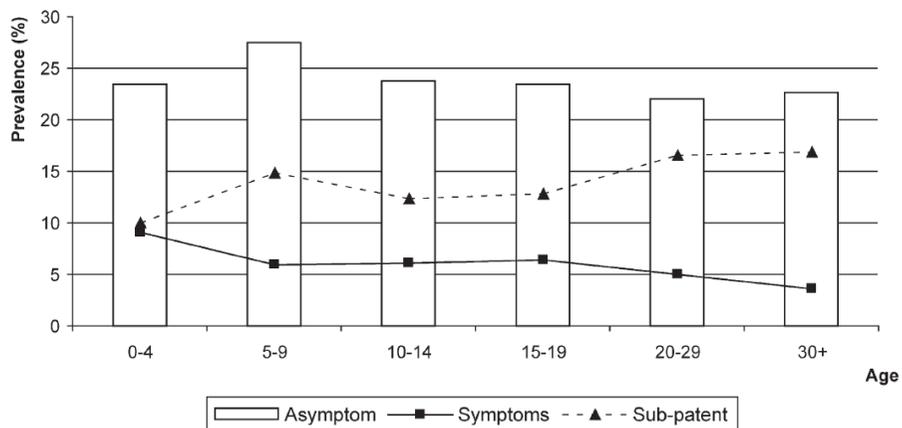


Figure 3. Prevalence of symptomatic, asymptomatic and sub-patent malaria infections by age group, central Vietnam

Discussion

In Vietnam, the malaria control program has successfully relied on microscopy diagnosis to reduce the burden of malaria. As previously reported, in the remaining endemic areas located mainly in central Vietnam, asymptomatic infections are common [1]. Our analysis by PCR showed a higher prevalence and more complex species distribution than expected by routine microscopy. Half of infections detected by PCR were sub-patent, suggesting that the actual occurrence of malaria exceeds that usually estimated by microscopy. This finding was reported in a meta-analysis by Okell and others in 2009, which showed that the prevalence of *P. falciparum* infection by microscopy was on average 50% of that measure by PCR and that this difference was increasing with decreasing transmission [15]. More precisely, the median parasite

prevalence by PCR was 35.7% (IQR=24.9-48.5) for areas where microscopic prevalence is 10.0-24.9%, which is consistent with our results, i.e., 29.1% and 14.4%, respectively.

More interestingly, *P. malariae* and *P. ovale* were not rare species, as detected by standard microscopy, and often occurred in mixed infections, which were commonly observed by PCR in our population. These findings confirmed previous reports from other countries in Southeast Asia and Africa, where use of molecular techniques showed a high complexity of infection, i.e., high proportions of mixed infections and high occurrence of *P. malariae* and *P. ovale* compared with that detected by standard microscopy [7, 8, 16-18].

Presumably, low parasite densities made it difficult to accurately identify the *Plasmodium* infection and to detect cryptic species by microscopy. This suggestion is supported by the higher parasite densities for the mixed infection identified by both techniques, and the constantly higher frequency of *P. falciparum* or *P. vivax* mono-infections identified at low level densities. Because *P. malariae* and *P. ovale* have so far been considered rare species, technicians are more likely to recall the well-known *P. falciparum* and *P. vivax* mono-infections. In cases of low parasite counts, the higher sensitivity and specificity of PCR over microscopy lead to more accurate species identification [7, 8, 16].

Because PCR cannot be routinely used for diagnosis, awareness of the species distribution and further training on the detection of non-falciparum malaria could improve the control efforts. This is particularly important for *P. vivax* and *P.*

ovale, for which dormant liver forms can be reactivated months or years after the primary infection, but also for *P. malariae* which has been reported to cause malaria attack years after the primary infection [19].

The high proportion of asymptomatic and sub-patent infections indicates the development of partial immunity that is able to control parasitaemia and maintain it at low and even undetectable densities [5, 20]. The degree of partial immunity can be estimated by analyzing the risk of infection by age groups under the assumption that it would increase with age, due to longer exposure. The effect of age on the risk of infection was adjusted for the confounding effects of forest activity, bed net use and socioeconomic level, which were shown to be strong risk factors for malaria infections, to get a better estimate of the independent effect of age [14]. Thus, the adjusted effect of age showed that the odds of infection decreased with increased age. In persons ≥ 20 years of age, the odds of infection were significantly lower compared with children less than five years of age. This age dependent risk was also illustrated by the evolution the ratio of sub-/patent infections. Patent infections were more common in the younger groups, and in persons ≥ 20 years of age sub-patent infections largely exceeded the patent infections. This finding was confirmed in the multivariate adjusted analysis where the odds of patent infection was reduced by $\geq 80\%$ and the odds of symptomatic infection was reduced by $\geq 66.7\%$ in infected adults compared with infected children. A recent report by Proietti and others showed similar results from an area of high malaria endemicity in Uganda, where the promotion of sub-

patient *P. falciparum* infections changed from 20% in children less than 15 years of age to approximately 50% in the older age groups [18].

With increasing age, the prevalence of gametocyte carriage and mixed infections decreased markedly and their risks were significantly decreased by 75% and 60% respectively, at the age of adolescence. The observed results of age-dependency likely indicate the progressive acquisition of immunity. Other reports mentioning the age-dependency of malaria prevalence and symptomatic disease also attributed this phenomenon to the acquisition of protective immunity [5, 8, 10, 20]. Two forms of immunity are likely to occur, one form is the anti-disease immunity, which is illustrated by the numerous asymptomatic infections even in young children and the significantly lower risk of symptomatic infection (compared with asymptomatic infection) in adults [20, 21]. The other form is immunity against the parasites, which develops more slowly, and results in lower prevalence with low densities infections in the adult population. The partial development of anti-parasite immunity might be responsible for lower risk of symptomatic and patent infection by age. The reduction of the risk for symptomatic and patent disease became significant in adults and corresponds to cumulative exposure during adult life when working in forest fields. In addition, as observed in Indonesia, immunity to infections may develop more rapidly in adults than in children, probably contributing to the lower risk of clinical malaria observed in individuals ≥ 20 years of age [22, 23].

Adults had lower gametocyte carriage, suggesting a lower transmissibility to the vector. However, the gametocytes identified by microscopy are likely to represent only a tip of the iceberg [24]. In Kenya, the gametocyte prevalence in children infected with *P. falciparum* was approximately four times higher by PCR, compared with that determined by microscopy [25]. The long duration of gametocytes carriage after infection (≤ 55 days) and the ability of sub-patent gametocyte infections to infect mosquitoes makes the adult population a potent reservoir for sustaining transmission [25, 26]. This suggestion is plausible if one considers the behavior of the vector (exophilic and exophagic) and exposure to mosquito bites of adults in the forest, where these persons can easily infect mosquitoes. The contribution of asymptomatic and sub-patent infection to maintain malaria transmission within this context should be further clarified. Sub-patent and asymptomatic infections have been shown to be infective to mosquitoes, although at lower rates than patent and symptomatic infections, but because sub-patent and asymptomatic infections are more prevalent and remain undetected longer, their contribution might be substantial [9].

If one considers that is a cross-sectional study, the evolution of asymptomatic or sub-patent infections towards clinical malaria cannot be excluded because infected persons were not followed-up and may have shown development of a clinical attack after the survey. Nevertheless, asymptomatic carriage, as indicated by its prevalence and by several previous reports for the region, is probably a common occurrence [1, 14, 24].

Therefore, the true parasite distribution of all five *Plasmodium* species infecting human in the malaria-endemic regions of Vietnam can be estimated only by conducting similar surveys in other districts or provinces to which malaria is endemic [27]. Such information will be essential for guiding and monitoring future elimination efforts.

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References

1. Thang ND, Erhart A, Hung le X, Thuan le K, Xa NX, Thanh NN, Ky PV, Coosemans M, Speybroeck N, D'Alessandro U: Rapid decrease of malaria morbidity following the introduction of community-based monitoring in a rural area of central Vietnam. *Malar J* 2009, *8*: 3.
2. Erhart A, Ngo DT, Phan VK, Ta TT, VAN Overmeir C, Speybroeck N, Obsomer V, Le XH, Le KT, Coosemans M, D'alessandro U: Epidemiology of forest malaria in central Vietnam: a large scale cross-sectional survey. *Malar J* 2005, *4*(1): 58.
3. Kawamoto F, Miyake H, Kaneko O, Kimura M, Nguyen TD, Nguyen TD, Liu Q, Zhou M, Le DD, Kawai S, Isomura S, Wataya Y: Sequence variation in the 18S rRNA gene, a target for PCR-based malaria diagnosis, in *Plasmodium ovale* from southern Vietnam. *J Clin Microbiol* 1996, *34*(9): 2287—2289.
4. Gleason NN, Fisher GU, Blumhardt R, Roth AE, Gaffney GW: *Plasmodium ovale* malaria acquired in Viet-Nam. *Bull World Health Organ* 1970, *42*(3): 399—403.
5. 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*(3): 452—459.
6. 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—648.
7. Steenkeste N, Rogers WO, Okell L, Jeanne I, Incardona S, Duval L, Chy S, Hewitt S, Chou M, Socheat D, Babin FX, Arieu F, Rogier C: Sub-microscopic malaria cases and mixed malaria infection in a remote area of high malaria endemicity in Rattanakiri province, Cambodia: implication for malaria elimination. *Malar J* 2010, *9*: 108.
8. Putaporntip C, Hongsrimuang T, Seethamchai S, Kobasa T, Limkittikul K, Cui L, Jongwutiwes S: Differential prevalence of *Plasmodium* infections and cryptic

- Plasmodium knowlesi* Malaria in Humans in Thailand. *J Infect Dis* 2009, 199: 1143—1150.
9. Alves FP, Gil LH, Marrelli MT, Ribolla PE, Camargo EP, Da Silva LH: Asymptomatic carriers of *Plasmodium spp.* as infection source for malaria vector mosquitoes in the Brazilian Amazon. *J Med Entomol* 2005, 42(5): 777—779.
 10. Coura JR, Suárez-Mutis M, Ladeia-Andrade S: A new challenge for malaria control in Brazil: asymptomatic Plasmodium infection--a review. *Mem Inst Oswaldo Cruz* 2006, 101(3): 229—237.
 11. Peeters Grietens K, Xuan XN, Van Bortel W, Duc TN, Ribera JM, Ba Nhat T, Van KP, Le Xuan H, D'Alessandro U, Erhart A: Low perception of malaria risk among the Ra-glai ethnic minority in south-central Vietnam: implications for forest malaria control. *Malar J* 2010, 9:23.
 12. Plowe CV, Djimde A, Bouare M, Doumbo O, Wellems TE: Pyrimethamine and proguanil resistance-conferring mutations in *Plasmodium falciparum* dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa. *Am J Trop Med Hyg* 1995, 52: 565—568.
 13. Rubio JM, Post RJ, van Leeuwen WM, Henry MC, Lindergard G, Hommel M: Alternative polymerase chain reaction method to identify *Plasmodium* species in human blood samples: the semi-nested multiplex malaria PCR (SnM-PCR). *Trans R Soc Trop Med Hyg* 2002, 96 (1 Suppl): S199--S204.
 14. Thang ND, Erhart A, Speybroeck N, Hung le X, Thuan le K, Hung CT, Ky PV, Coosemans M, D'Alessandro U: Malaria in central Vietnam: analysis of risk factors by multivariate analysis and classification tree models. *Malar J* 2008, 7: 28.
 15. Okell LC, Ghani AC, Lyons E, Drakeley CJ: Submicroscopic infection in *Plasmodium falciparum*-endemic populations: a systematic review and meta-analysis. *J Infect Dis* 2009, 200:1509-1517.
 16. Coleman RE, Sattabongkot J, Promstaporm S, Maneechai N, Tippayachai B, Kengluecha A, Rachapaew N, Zollner G, Miller RS, Vaughan JA, Thimasarn K,

- Khuntirat B: Comparison of PCR and microscopy for the detection of asymptomatic malaria in a *Plasmodium falciparum/vivax* endemic area in Thailand. *Malar J* 2006, 5: 121.
17. Oguike MC, Beston M, Burke M, Nolder D, Stothard JR, Klienschmidt I, Proietti C, Bousema T, Ndounga M, Tanabe K, Ntege E, Culleton R, Sutherland CJ: *Plasmodium ovale curtisi* and *Plasmodium ovale wallikeri* circulate simultaneously in African communities. *Int J Parasitol* 2011, 41:677-683.
 18. Proietti C, Pettinato DD, Knoi BN, Ntege E, Crisanti A, Riley EM, Egwang TG, Darkeley C, Bousema T: Continuing intense malaria transmission in northern Uganda. *Am J Trop Med Hyg* 2011, 84:830-837.
 19. Collins WE, Jeffery GM: *Plasmodium malariae*: parasite and disease. *Clin Microbiol Rev* 2007, 20(4): 579—592.
 20. Mayor A, Aponte JJ, Fogg C, Saúte F, Greenwood B, Dgedge M, Menendez C, Alonso PL: The epidemiology of malaria in adults in a rural area of southern Mozambique. *Malar J* 2007, 6: 3.
 21. Doolan DL, Dobaño C, Baird JK: Acquired immunity to malaria. *Clin Microbiol Rev* 2009, 22(1): 13—36.
 22. Baird JK, Purnomo, Basri H, Bangs MJ, Andersen EM, Jones TR, Masbar S, Harjosuwarno S, Subianto B, Arbani PR: Age-specific prevalence of *Plasmodium falciparum* among six populations with limited histories of exposure to endemic malaria. *Am J Trop Med Hyg* 1993, 49(6): 707—719.
 23. Baird JK, Krisin, Barcus MJ, Elyazar IR, Bangs MJ, Maguire JD, Fryauff DJ, Richie TL, Sekartuti, Kalalo W: Onset of clinical immunity to *Plasmodium falciparum* among Javanese migrants to Indonesian Papua. *Ann Trop Med Parasitol* 2003, 97(6): 557—564.
 24. Erhart A, Ngo DT, Phan VK, Ta TT, Van Overmeir C, Speybroeck N, Obsomer V, Le XH, Le KT, Coosemans M, D'alessandro U: Epidemiology of forest malaria in central Vietnam: a large scale cross-sectional survey. *Malar J* 2005, 4: 58.

25. Bousema JT, Schneider P, Gouagna LC, Drakeley CJ, Tostmann A, Houben R, Githure JJ, Ord R, Sutherland CJ, Omar SA, Sauerwein RW: Moderate effect of artemisinin-based combination therapy on transmission of *Plasmodium falciparum*. *J Infect Dis* 2006, *193*(8): 1151—1159.
26. Bousema T, Okell L, Shekalaghe S, Griffin JT, Omar S, Sawa P, Sutherland C, Sauerwein R, Ghani AC, Drakeley C: Revisiting the circulation time of *Plasmodium falciparum* gametocytes: molecular detection methods to estimate the duration of gametocyte carriage and the effect of gametocytocidal drugs. *Malar J* 2010, *9*: 136.
27. Van den Eede P, Van HN, Van Overmeir C, Vythilingam I, Duc TN, Hung le X, Manh HN, Anné J, D'Alessandro U, Erhart A: Human *Plasmodium knowlesi* infections in young children in central Vietnam. *Malar J* 2009, *8*: 249.

Chapter 6

Population genetics of *Plasmodium vivax* in four rural communities in Central Vietnam

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Abstract

Background: The burden of malaria in Vietnam has drastically reduced, prompting the National Malaria Control Program to officially engage in elimination efforts. *Plasmodium vivax* is becoming increasingly prevalent, remaining a major problem in the country's central and southern provinces. A better understanding of *P. vivax* genetic diversity and structure of local parasite populations will provide baseline data for the evaluation and improvement of current efforts for control and elimination. The aim of this study was to examine the population genetics and structure of *P. vivax* isolates from four communities in Tra Leng commune, Nam Tra My district in Quang Nam, Central Vietnam.

Methodology/Principal Findings: *P. vivax* mono infections collected from 234 individuals between April 2009 and December 2010 were successfully analyzed using a panel of 14 microsatellite markers. Isolates displayed moderate genetic diversity ($H_e = 0.68$), with no significant differences between study communities. Polyclonal infections were frequent (71.4%) with a mean multiplicity of infection of 1.91 isolates/person. Low but significant genetic differentiation (F_{ST} value from -0.05 to 0.18) was observed between the community across the river and the other communities. Strong linkage disequilibrium ($I_A^S = 0.113$, $p < 0.001$) was detected across all communities, suggesting gene flow within and among them. Using multiple approaches, 101 haplotypes were grouped into two genetic clusters, while 60.4% of haplotypes were admixed.

Conclusions/Significance: In this area of Central Vietnam, where malaria transmission has decreased significantly over the past decade, there was moderate genetic diversity and high occurrence of polyclonal infections. Local human populations have frequent social and economic interactions that facilitate gene flow and inbreeding among parasite populations, while decreasing population structure. Findings provide important information on parasites populations circulating in the study area and are relevant to current malaria elimination efforts.

Author Summary

In Vietnam, *Plasmodium vivax* (*P. vivax*) is the second most frequent human malaria parasite and a major obstacle to countrywide malaria elimination. Knowing the local parasite structure is useful for elimination efforts. Therefore, we analyzed, with a panel of 14 microsatellite markers, 234 *P. vivax* mono infections in blood samples collected from 4 communities in central Vietnam. Genetic diversity in the population was moderate; a high occurrence of polyclonal infections and significant linkage disequilibrium were detected, suggesting inbreeding or recombination between highly related haplotypes. In addition, both genetic differentiation and population structure was low and only detected between communities at each side of the river. Those results suggest gene flow between study communities with the river defining a moderate geographical barrier. Future studies should determine how this genetic variation is maintained in an area of extremely low transmission.

Introduction

Vietnam has been extremely successful in decreasing the country's malaria burden, thanks to the large scale implementation of control interventions such as insecticide-treated bed nets, indoor residual spraying, and prompt, free-of-charge diagnosis and treatment; the number of cases fell from 130,000 in 2004 to 27,868 in 2014 [1]. Malaria has been virtually eliminated from Northern and Southern Vietnam [1, 2]. In 2014, 80% of malaria cases occurred in nine "hot provinces" where annual incidence peaked at 3.1 cases per 1000, indicating a highly heterogeneous transmission, with hot spots of transmission (mostly in mountainous and forested areas) surrounded by areas of low transmission [1-5]. Vietnam aims at eliminating malaria by 2030 [6]. Such ambitious goal is threatened by *P. vivax*, whose characteristics (dormant liver forms that relapse weeks or months after clearance of the primary infection and gametocytes production before the occurrence of symptoms) together with the relative high occurrence of sub-patent and asymptomatic infections that remain undetected and thus untreated [3, 7-10], make its transmission much more difficult to interrupt than that of *P. falciparum*. In addition, as already reported, elimination efforts are threatened by the emergence of drug resistance, for *P. falciparum* to artemisinin derivatives and partner drugs and for *P. vivax* to chloroquine (CQ) [2, 11, 12].

Current efforts to eliminate malaria are targeted to districts and communes reporting an increased number of malaria cases over time [2, 6]. In this context, understanding parasite genetic diversity and its population structure is relevant for (i) monitoring

temporal changes in transmission following control efforts, (ii) elucidating the spatial distribution of parasite populations and predicting outbreaks, population resilience, and the spread of drug-resistant parasites, and (iii) identifying ecological and behavioral risk factors that can inform malaria control and elimination efforts [13-15].

A previous study conducted in Binh Thuan province in central Vietnam reported high levels of genetic diversity (average expected heterozygosity (He) = 0.86) and all infections being multi-clonal despite low transmission [13] (similar to what has been previously reported in South-East Asia) [16].

The aim of this study was to provide baseline data on the *P. vivax* parasite populations in four rural communities in the Vietnamese Quang Nam province.

Materials and methods

Study site

Samples were collected from April 2009 to December 2010 in four communities (Figure 1) in the South Tra My district of Quang Nam, Central Vietnam during a prospective cohort study aiming to assess the short- and long-term efficacy of CQ and high-dose piperaquine (PQ) for the treatment of *P. vivax* mono-infections [12]. Detailed sociodemographic characteristics of the local population have been already reported elsewhere [3]. In 2009, the prevalence of malaria by light microscopy was 7.8%, while by polymerase chain reaction (PCR) prevalence was estimated at 22.6% (ranging from 16.4 to 42.5%), with a high proportion of *P. vivax* mono infections (43%). Sub-patent

infections accounted for 58.7% of all infections, evidencing the existence of a substantial hidden human reservoir of malaria [3].

Malaria transmission is seasonal, and peaks during the rainy season (May to November). Based on data from the Provincial Malaria Station, between 2009 and 2013 the mean prevalence of *P. falciparum*, *P. vivax*, and mixed malaria cases for all age groups in the study area was 64.1%, 31.5%, and 4.4%, respectively [17-18]. The main malaria vectors in the area are *Anopheles dirus sensu stricto* and *An. minimus*, though *An. vagus*, *An. aconitus*, and *An. philippinensis* are also present [17-18].

Sample collection

A finger prick blood sample was collected at day 0 (before treatment) for diagnosis by light microscopy and two blood spots were collected on grade 3 filter paper (Whatman Ltd., Springfield Mill, Maidstone, United Kingdom) for molecular diagnosis and microsatellite (MS) genotyping.

Ethics statement

The study was approved by the National Institute of Malariology, Parasitology and Entomology in Hanoi, the Ministry of Health of Vietnam, and the review boards of the Institute of Tropical Medicine and Antwerp University Hospital (UZA) in Antwerp, Belgium. Adult participants (in case of minors one of the parents/guardians) provided written informed consent.

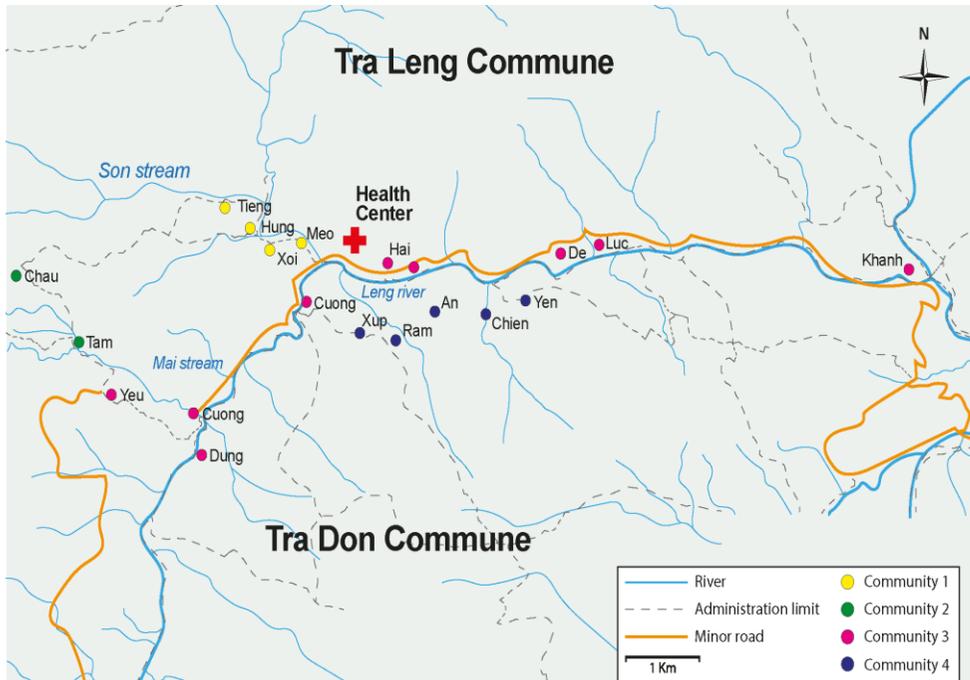


Figure 1. Study area in Nam Tra My district, Quang Nam, Vietnam

Light microscopy diagnosis

Thick and thin film blood slides were stained with a 3% Giemsa solution for 45 minutes, and the number of asexual parasites was calculated following World Health Organization (WHO) guidelines [19]. Parasite density was estimated by dividing the number of asexual parasites for 200 white blood cells (WBCs) counted and expressed as the number of asexual parasites per microliter of blood, assuming 8000 WBC/ μ L. All blood slides were double-read by two technicians and in case of disagreement; slides were read by a third senior technician. The final results were expressed as the

mean of the two closest results. A slide was declared negative if no parasites were found after counting 1,000 WBCs.

Genotyping

DNA was extracted from filter paper blood spots cut into 5-mm-diameter disks with the QIAamp DNA Micro Kit following the manufacturer's recommendations (Qiagen, Hilden Germany). *P. vivax* mono infections were confirmed by species-specific multiplexed semi-nested PCR, as described by Rubio et al. [20]. All samples were genotyped with 14 MS (MS1-MS10, MS12, MS15, MS20, and PvSal1814) following previously described PCR protocols [13-14]. The PCR products of four MS were pooled and analyzed by capillary electrophoresis in a 3730 XL ABI sequencer (Applied Biosystems) and 1200 Liz was used as the internal size standard. Negative samples were repeated once.

Allele calling was performed using GeneMarker version 2.4.0. After pooling capillary electrophoresis fsa files from all samples, a standard cut-off value of 500 relative fluorescence units was defined and peaks below this limit were considered background noise. In addition, all samples were double-checked manually to confirm true alleles. At each locus the predominant allele (the one giving the highest peak) and minor alleles within at least two-thirds of the height of the predominant allele were scored [13, 15, 21]. Only predominant alleles were used to define haplotypes to ensure an unbiased estimate of minor allele frequency in polyclonal infections [15, 22-24].

Data analysis

Samples were defined as polyclonal if at least one locus presented more than one allele [13-14]. Polyclonal/locus (%) describes the percentage of samples identified as polyclonal by a given MS out of the total number of samples [13]. Multiplicity of infection (MOI), defined as the minimum number of different clones observed in a sample, was estimated by taking the maximum number of alleles at the two most polymorphic markers [13, 25]. Average MOI was defined as the sum of MOIs detected across all samples divided by the total number of samples. Average MOI and the proportion of monoclonal and polyclonal infections were compared with the Kruskal-Wallis and Pearson χ^2 test, respectively. A value of $p < 0.05$ was considered significant. The predominant alleles in each sample were used to calculate the number of haplotypes by GenAEx 6.5 [26]. Haplotypes that appear only once in the population were defined as unique haplotypes.

Genetic diversity, defined as the probability of observing different genotypes at a given locus in two unrelated parasites, was assessed by calculating the expected heterozygosity (H_e) for each community using the formula: $H_e = [n/(n-1)]*(1-\sum p_i^2)$, where n is the total number of alleles and p is the allele frequency. H_e ranges between 0 and 1, with values close to 1 indicating high genetic diversity [27]. Allelic richness, defined as the number of alleles per locus independently of sample size, was calculated using FSTAT v2.9.3 [28]. H_e and allelic richness were compared between communities using the Kruskal-Wallis test. The presence of bias due to false

assignment of predominant haplotypes was investigated by comparing He in the database containing all the alleles and the database containing the predominant alleles [29].

A standardized index of association (I_A^s), calculated with LIAN v3.5, was used to assess the presence of multilocus linkage disequilibrium (LD) in the parasite population [30]. The significance of the I_A^s estimate was assessed with Monte Carlo simulation using 10,000 random permutations of the data. To differentiate between clonal propagation and epidemic expansion, we compared LD in the predominant allele dataset and the unique haplotype dataset [31]. Pairwise LD was used to evaluate the physical linkage between loci located within the same contig using the G statistic in FSTAT v2.3.9 [28, 32].

Genetic differentiation between pairs of communities was estimated using a pairwise unbiased estimator of F-statistics FSTAT v2.3.9 with no assumption of Hardy-Weinberg equilibrium within samples [28, 32]. A matrix of p values corresponding to each pairwise F_{ST} was calculated after Bonferroni correction, with a value of $p < 0.05$ considered significant. Crude F_{ST} values were adjusted for sample size using Recode Data v. 0.1 [33] and standardized F_{ST} estimates were obtained by dividing crude F_{ST} values by adjusted F_{ST} values. F_{ST} estimates ranged from 0 (no genetic differentiation between communities) to 1 (full differentiation).

As a complementary approach, population structure was investigated using the software programs STRUCTURE v2.3.2 [34], CLUMPP [35], DISTRICT [36], and

GENODIVE [37]. STRUCTURE was used to identify clusters of genetically related samples. The number of clusters (K) was set from 1 to 10 with 10 replications per K , and 150,000 Markov Chain Monte Carlo steps after a burn-in period of 50,000 iterations using the admixture model. The loc-prior model was used for accurate inference of population and individual ancestry. Next, we used STRUCTURE HARVESTER v0.6.94 [38] to calculate the most likely number of K clusters. Additional data parsing and formatting of the STRUCTURE output was performed using CLUMPP and DISTRUCT [38-39]. CLUMPP permutes the clusters' output by performing multiple replicate runs for the selected K . Samples with an average pairwise similarity (H value) of over 85% in one of the K clusters were considered to belong to that particular population; all other samples were considered admixed samples. DISTRUCT performs geographical displays of the aligned cluster assignment. We confirmed the optimal K , i.e. the K with the highest pseudo-F statistic, using AMOVA-based K -means clustering analysis in GENODIVE V2.0b23 (OS X 10.6 operating system). This method divides a number of individuals into an a priori assigned number of clusters (K) in such a way that minimizes within-group diversity and maximizes between-group diversity. The pseudo-F statistic was calculated by setting up simulated annealing runs with 150,000 steps and 50 algorithm repetitions to determine optimal clustering (highest pseudo-F-statistic).

eBURST v3 was used to identify clusters of closely related haplotypes, or haplogroups (HG), which were defined as haplotypes sharing at least 9 loci from the 14 MS

analyzed [40]. Haplotypes unrelated to any haplogroup (HG) were classified as singletons. The relationship between haplotypes following the defined K clusters was further analyzed by PHYLOViz [41]. Finally, to investigate the relationship between geographic and genetic distances in the study population, we performed principal coordinate analysis with the Mantel test for matrix correspondence in GenAlEx 6.5 [26]. Allele frequency was calculated in GenAlEx 6.5 using the predominant allele data set with 14 loci. The existence of a recent population bottleneck was investigated by evaluating the allele frequency distribution in the population (alleles at low frequencies are less abundant in populations with a recent bottleneck) [42-43].

Results

Baseline characteristics

In total, 234 individuals with *P. vivax* mono infection from the 260 recruited in the original study [12] were successfully genotyped and included in the analysis. Baseline characteristics of study participants are described in Table 1.

Table 1. Baseline characteristics of study patients

Community	Sample	EM	Age groups		Sex		Hamlets	HH	Season of collection		AS	MP	MG
			≤15y	>15 y	M	F			Dry	Rainy			
Community 1 (%)	92 (39.3)	Cadong	55 (23.5)	37 (15.8)	51 (21.8)	41 (17.5)	4 (28.6)	45 (36.3)	16 (6.8)	76 (32.5)	59 (25.2)	7872	652
Community 2 (%)	57 (24.4)	Cadong	34 (14.5)	23 (9.8)	35 (15.0)	22 (9.4)	2 (14.2)	30 (24.2)	11 (4.7)	46 (19.7)	36 (15.4)	5829	1162
Community 3 (%)	32 (13.7)	Cadong	26 (11.1)	6 (2.6)	21 (8.9)	11 (4.7)	5 (35.8)	23 (18.5)	5 (2.1)	27 (11.5)	19 (8.1)	7144	490
Community 4 (%)	53 (22.6)	M'nong	29 (12.4)	24 (10.3)	33 (14.1)	20 (8.5)	3 (21.4)	26 (21.0)	6 (2.6)	47 (20.1)	24 (10.3)	5297	516
Total (%)	234 (100)		144 (61.5)	90 (38.5)	140 (59.8)	94 (40.2)	14 (100)	124 (100)	38 (16.2)	196 (83.8)	138 (59.0)	6691	723

EM, ethnic minority; HH, household; AS, asymptomatic infection (defined as no fever at enrolment), MP, mean number of asexual parasites per μL ; MG, mean number of gametocytes per μL .

Genetic diversity

Successful genotyping, with at least 12 of the 14 MS, was achieved in 194 patients (82.9%). Allele data were successfully recovered in more than 83% of the samples for all MS except MS20 and Pvsal1814, for which successful amplification was achieved in 66% and 75% of samples respectively.

The MS characteristics are described in Table 2. Overall, genetic diversity was moderate, with an average $He = 0.68$ (95%CI 0.58–0.77) for all MS. MS3 and MS9 were the least polymorphic markers ($He = 0.45$ and 0.31 respectively), while MS10 and Pvsal1814 were the most polymorphic markers ($He = 0.99$ and 0.92 respectively), which were therefore used to calculate MOI. The number of alleles per MS ranged from 3 to 14. All MS had non-significant differences in He values in the database containing all alleles per locus and the predominant allele datasets ruling out bias in the construction of haplotypes from polyclonal infections ($p=0.68$). The average number of alleles per locus was 5.5 (95%CI 3.82–7.17), the average number of alleles detected in a sample by any locus was 1.14 (95%CI 1.0–1.27) and the average allelic richness was 5.05 (95%CI 4.11–5.98).

The proportion of polyclonal infections, similar in all communities ($p > 0.05$), was 71.4% (167/234) when all 14 MS were used, but 64.1% (141/220) ($N = 220$ as 14 samples had missing data for MS10 and Pvsal1814) when only Pvsal1814 and MS10, the most polymorphic markers were used. The same two MS were used to calculate MOI (based on 220 samples with completed data), whose mean in the four communities was 1.91

(95% CI 1.81–2.02), with no significant differences between communities ($p = 0.52$), age groups ($MOI_{\leq 15\text{years}} = 2.0$ vs $MOI_{>15\text{years}} = 1.9$, $p = 0.50$), gametocyte carriage ($MOI_{\text{gametocytes present}} = 1.93$ vs $MOI_{\text{gametocytes absent}} = 1.80$, $p = 0.42$), sex ($MOI_{\text{male}} = 1.89$ vs $MOI_{\text{female}} = 1.95$, $p = 0.55$), symptomatic vs asymptomatic (defined as fever at enrolment vs no fever at enrolment, $MOI = 1.98$ vs $MOI = 1.80$, respectively) ($p = 0.10$), season ($MOI_{\text{rainy season}} = 1.90$ vs $MOI_{\text{dry season}} = 1.97$, $p = 0.64$), and ethnic minority ($MOI_{\text{Cadong}} = 1.89$ vs $MOI_{\text{M'nong}} = 2.0$, $p = 0.46$). No significant differences were found between the four communities for either level of He ($p = 0.08$) or allelic richness ($p = 0.31$).

We identified 101 haplotypes from 144 samples of which 84 haplotypes were defined as unique haplotype with complete genotyping data for 13 MS. MS20 was excluded because it had the lowest successful genotyping rate (66%). Of these haplotypes, 25.7% (26/101) were found in monoclonal infections and 16.8% (17/101) were found in both monoclonal and polyclonal infections; 6.93% of haplotypes (7/101) had a frequency of over 2 in 40 samples and the two most frequent haplotypes were detected in 7.6% (11/144) and 6.2% (9/144) of samples. One haplotype was shared between the four communities, 4 haplotype found in community 1 were also present in community 2 and one haplotype shared between community 3 and community 4.

Table 2. Characteristics of 14 microsatellite loci used in *P. vivax* populations from four communities in Quang Nam, Vietnam (n = 234)

Locus	Repeat sequence	ASR	No of alleles	H_E	A*	PI/locus (%)	hMOI	Average alleles/ locus
MS1	(GAA)11	211-239	5	0.664	4.94	3.41	3	1.04
MS2	(TAAA)2TATA (TAAA)6 TATA (TAAA)19	179-215	4	0.686	4.00	5.12	2	1.06
MS3	(GAA)11	185-199	3	0.446	3.00	4.27	2	1.11
MS4	(AGT)18	189-237	6	0.728	5.94	20.94	3	1.24
MS5	CCTCTT(CCT)11	164-206	5	0.697	4.94	1.70	2	1.01
MS6	(TCC)2(TCT)3(CCT)2(TTC)2 GCTTCT(TCC)10	242-254	5	0.655	5.00	5.55	2	1.06
MS7	(GAA)9	142-157	3	0.601	3.00	5.55	2	1.06
MS8	(CAG)2(CAA)11	198-268	6	0.703	5.94	5.55	2	1.06
MS9	(GGA)18	155-170	3	0.311	3.00	2.56	2	1.03
MS10	GAA(GGA)2AGA(GGA)9AGA(GGA)4AGAG GAAGA(GGA)3AGAGGAAGA(GGAAAA)4(GGA)2 (AGA)11(GGA)3(AGA)2GGAAGA(GGA)2	184-222	9	0.993	8.94	9.82	3	1.12
MS12	(TTC)10(TGC)4	207-224	5	0.618	5.00	5.55	2	1.06
MS15	(TCT)10	241-281	5	0.525	5.00	5.12	2	1.05
MS20	(GAA)11GAG(GAA)13(CAA)4GAA(CAA)5	148-206	4	0.680	4.00	3.41	2	1.05
Pvsal 1814	(AGA)44	546-704	14	0.919	7.99	53.84	3	2.01
All samples		142-704	77	0.676	5.05	71.37	2.2	1.14

ARS, Allele size range; A, Allelic richness; hMOI, highest multiplicity of infection; PI, Polyclonal infection. * Predominant alleles only.

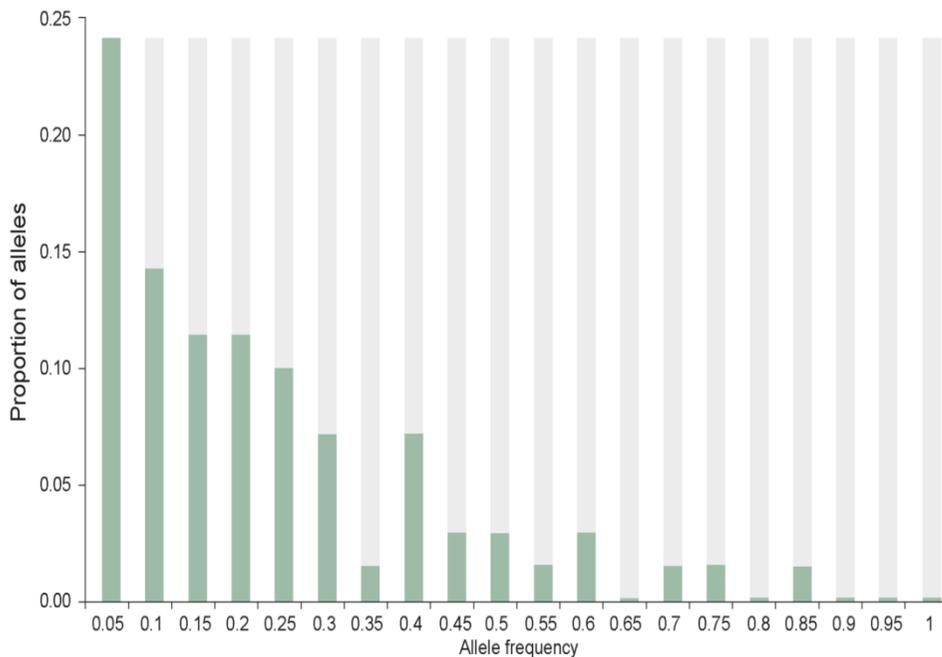


Figure 2. Allele frequency distribution in four communities in Quang Nam (n = 234)

Predominant allele database and all loci combined are included.

To evaluate the existence of a recent population bottleneck we analyzed the allele frequency distribution in the population. Figure 2 shows an L-shaped distribution of allele frequencies, as would be expected from neutral evolution.

Linkage disequilibrium

MS20 was also excluded from the LD analysis to maximize sample size and avoid bias due to an imbalanced number of samples between communities. Hence at least 25% of samples per community were included in the analysis. Significant LD was observed in each community (I_A^S ranged from 0.10 to 0.17) and in the overall study population ($I_A^S = 0.113$, $p < 0.001$). LD remained significant ($I_A^S = 0.059$, $p < 0.001$) when only the unique haplotypes were used. We then examined patterns of LD between pairs of MS

(Figure 3). Pairwise LD was observed between loci located within the same contigs (MS4-MS5, MS7-MS8, and MS12-MS15) and also within different contigs. Even though lower pairwise LD was observed in communities 3 and 4 compared to 1 and 2, the fact that the overall LD was significant ($p = 0.008$) suggests the existence of a clonal parasite population.

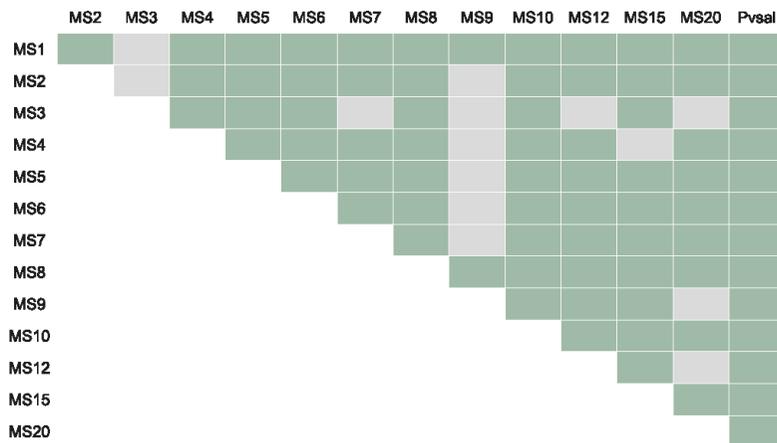


Figure 3. Pairwise loci linkage disequilibrium analysis (N = 234).

Significant association between alleles at pairs of loci in each sample was tested using FSTAT. Green color denotes LD at 5% based on 36400 permutations. Markers within the same contig are: MS4-MS5, MS7-MS8, MS12-MS15.

Genetic differentiation

We first compared the datasets containing only monoclonal infections ($n = 67$) with the predominant allele ($n = 234$) and found low genetic differentiation ($F_{ST} = 0.05$), indicating absence of bias. Then, we calculated F_{ST} values for pairwise genetic differentiation between the four communities (Table 3). We observed moderate genetic differentiation between community 4 and the other communities ($F_{ST} = 0.15$ -

0.18) and low differentiation for the other combinations ($F_{ST} < 0.1$) ($n = 144$), indicating that the parasite population in community 4 is moderately, although significantly, differentiated from parasite populations in communities 1, 2, and 3 ($p = 0.008$). Similar F_{ST} values were obtained when only unique haplotypes ($n = 84$) were used.

Table 3. Pairwise genetic differentiation

Standardize F_{ST}	Community 1	Community 2	Community 3	Community 4
Community 1		0.046*	-0.028	0.153*
Community 2	0.043		-0.005	0.176*
Community 3	0.026	-0.035		0.168*
Community 4	0.174*	0.080*	0.145*	

The upper-right section shows the standardized fixation index (F_{ST}) values obtained from 13 microsatellite markers ($n = 144$, MS20 excluded). The lower-left section shows F_{ST} values obtained from unique haplotypes from 13 microsatellite markers ($n = 84$). * P value < 0.05

Population structure

Structure analysis identified the most likely clusters in the population to be (i) $K = 7$ ($\Delta K = 9.4$), (ii) $K = 2$ ($\Delta K = 5.2$), and (iii) $K = 3$ ($\Delta K = 3.2$) ($n = 144$). The AMOVA-based K -means clustering analysis identified $K = 2$ as the optimal number of clusters (pseudo- $F = 30.5$). We further analyzed the parasite population divided by $K = 2$ (cluster 1 and cluster 2) with CLUMPP and DISTRUCT (Figure 4), and found that 33.3% (48/144) of the samples (with complete haplotypes) observed in the study population belonged to cluster 1 and 20.1% (29/144) belonged to cluster 2 and 46.6% (67/144) were admixed

samples. Community 4 had the highest proportion of admixed samples (62.1%), followed by community 3 (53.9%), while community 1 and 2 had similar rates (40.9% and 41.3% respectively). The proportion of admixed samples remained high when the number of clusters was set to $K = 3$ and $K = 7$ (48.6% and 38.9%, respectively). Of note, cluster 1 samples were absent from community 4, which supports a moderate degree of population structure between communities 1-3 and community 4. However, principal coordinate analysis failed to detect geographical clustering (per community) in the population.

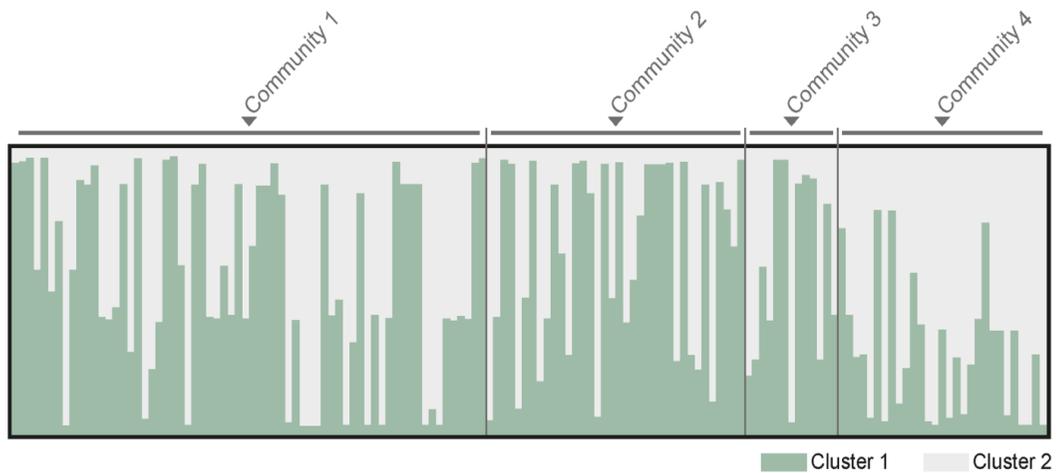


Figure 4. Genetic clustering analysis by STRUCTURE

The graph represent the clustering model when the parasite population was grouped into 2 clusters ($K = 2$). In the bar plot, each isolate is represented by a single vertical line divided into K colors.

Then, we investigated genetic relatedness, defined as haplotypes sharing at least 10/13 loci ($n = 101$) by eBURST. Eight different HGs and 13 singletons were identified. HG1 contained 51.5% (52/101) of all haplotypes in the four communities, while the

other 7 HGs contained between 2.0% (2/101) to 11.9% (12/101) haplotypes. However, when relatedness was defined as haplotypes sharing at least 9/13 loci, only 2 HGs and 2 singletons were identified. HG1 included 95.0% (96/101) of all haplotypes detected. PHYLOVIZ analysis supported the existence of related haplotypes among all study communities (with slightly clustering of community 4 samples) (Figure 5A) and confirmed the absence of cluster 1 haplotypes in community 4 (Figure 5B).

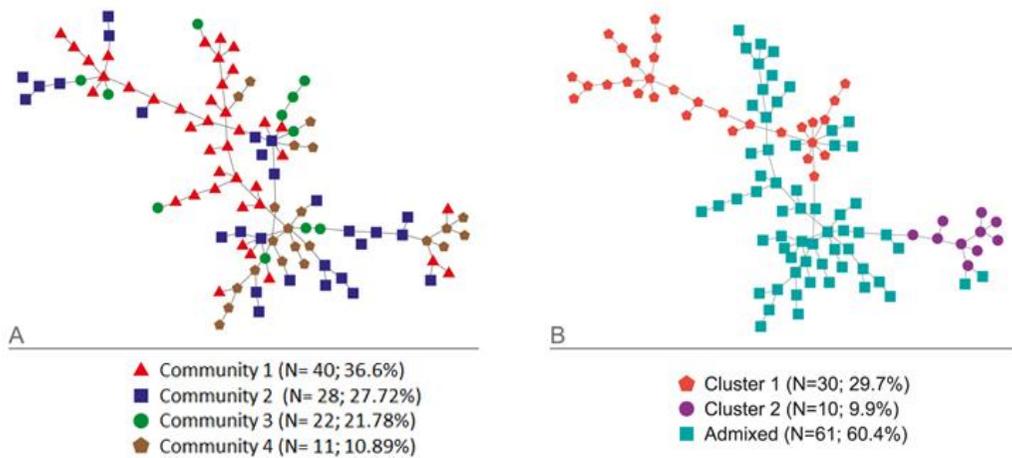


Figure 5. Phylogenetic relationship of 101 unique *P. vivax* haplotypes using PHYLOViZ.

Haplotypes relationships by (A) community, and (B) clustering K = 2. Each colored figure represents a unique haplotype and grey lines indicate shared alleles among individual haplotypes.

Discussion

We analyzed the genetic diversity and population structure of 234 *P.vivax* pre-treatment clinical isolates collected in a forested area of Central Vietnam between April 2009 and December 2010 [12]. We observed moderate levels of heterozygosity

in all four study communities, with a high proportion of polyclonal infections and significant LD, suggestive of inbreeding across parasite populations circulating in the study communities. Genetic differentiation and population structure between study communities was low but present between villages at each side of the river defining a moderate geographical barrier to gene flow.

In this study we used eight MS (MS1, MS4, MS6, MS9, MS10, MS12, MS15, and MS20) with balanced diversity, three (MS2, MS5 and Pvsal1814) with unbalanced diversity, one (MS8) with significant excess diversity, and two (MS3 and MS7) with significant reduced diversity [25]. Mean He in the study population ($He = 0.68$) using those 14 MS was non-significantly different to He when only MS with balanced diversity (recommended for measuring population diversity) were used. Therefore, all MS were kept in the analysis to assess both diversity parameters and polyclonal infections, but only the two most polymorphic markers (MS10 and Pvsal1814) were kept to investigate MOI.

The mean He in our study population ($He = 0.68$) was similar to figures seen in areas of north-west Brazil with similar transmission intensities ($He = 0.74$ and $He = 0.68$) [43-44]; higher than those observed in South Korea ($He = 0.43$) [45] and the Loreto district, Peru ($He = 0.37$) [16], and lower than those seen in Sri Lanka ($He = 0.89$) [46], Pursat, Cambodia ($He = 0.84$) [16], and Binh Thuan, Central Vietnam ($He = 0.88$) [13]. In our study MS9, MS3, and MS7 displayed the lowest number of alleles per locus ($n = 3$) and MS9 and MS3 had the lowest He values ($He_{MS9} = 0.31$ and $He_{MS3} = 0.45$). MS3, MS7, and

MS9 would therefore appear to be poorly informative markers in the study area and their use in future studies is not recommended.

Polyclonal infections were frequent (71.4%) when the results from 14 MS were combined and moderately lower (64.1%) when just Pvsal1814 and MS10 were used [47]. Mean MOI was 1.91, with similar MOI observed in symptomatic and asymptomatic study participants, possibly because of the high parasite density (mean 3,919/ μ L; 95%CI 2,852–4,986) detected in asymptomatic participants at day 0. In the literature, the proportion of polyclonal infections vary considerably depending on the MS markers used [16, 48], highlighting the need for a standardized methodology that allows comparison between studies and geographical regions. High proportions of polyclonal infections have also been reported in hypo-endemic areas in Sri Lanka (60%) [49], Colombia (60–80%) [48], the Amazon Basin in Brazil (50%) [43], and more recently, in a pre-elimination context in Sri Lanka (69%) [46]. It is noteworthy in a study carried out (1999-2000) in Binh Thuan province, central-south Vietnam, where the entomological inoculation rate was estimated at 1 infective bite/person/year, 100% of vivax infections were polyclonal with a mean MOI of 3.7 [13]. The high levels of genetic diversity and polyclonal infections in low transmission areas [13,23,48] can be, at least partially, explained by the unique biology of *P. vivax* which result in (i) a high prevalence of asymptomatic and low parasite density infections (which last longer because are difficult to detect, increasing the likelihood of repeated infections with divergent clones, resulting in increased polyclonality) and (ii) relapse from dormant liver stages

(the reactivation of heterologous clones increases the likelihood of peripheral superinfections). Since a high proportion of study participants were asymptomatic at recruitment (59.0%) and poor adherence to PQ radical cure is known in the study area [3], the high proportion of polyclonal infections found in this study may reflect peripheral superinfection fed by heterologous clones from both relapses and reinfections.

Despite those high rates of polyclonal infections, we observed a significant LD ($I_A^S = 0.113$, $p < 0.001$) in the overall study population. Asexual clones present in one infection produce gametocytes that, taken by the vector, recombine during meiosis and generate new haplotypes in a process known as outcrossing. Consequently, the breakdown of pre-existing associations between unlinked loci would reduce LD to low levels [50] as opposed to recombination between gametes from the same parasite [51]. As transmission decreases, fewer parasite types will be present in the population and recombination will often occur between related parasites, increasing the level of inbreeding in the population. This is supported by the fact that 53.8% of all polyclonal infections were identified by multiple alleles at just one locus. Indeed, LD remained significant in the analysis using only unique haplotypes, indicating that it is a result of inbreeding rather than expansion of few haplotypes due to outbreaks or epidemics [31]. Closely related parasites in hypoendemic areas have been previously reported [52, 53]. In addition, inbreeding was further supported by overall significant pairwise LD [21, 31]. LD combined with high levels of polyclonality has been reported

in rural Amazonia [54] and more recently in Sri Lanka [46]. The authors of these studies offered two alternative interpretations for this phenomenon. First, the MS may not be strictly neutral (10/14 MS mapping to loci encoding either hypothetical or annotated proteins may be subject to natural selection) [22-23]. And second, replication-slippage events during mitotic (asexual) replication could result in the generation of new alleles due to the addition or deletion of repeats [49, 55]. If the replication-slippage rate is higher than that of effective recombination (the probability of producing a recombinant genome), the clones generated would increase polyclonality, without altering LD.

It has been previously reported that replication-slippage events (and therefore number of alleles per locus and H_e) correlate positively with increasing repeat length and non-perfect repeats motifs, i.e. interrupted or compound motifs [25, 56]. Pvsal1814 MS used in this study, which had an $(AGA)_{44}$ motif structure with an interrupted/compound motif, $H_e = 0.91$ and 14 different alleles with frequencies ranging from 1.3% to 16%, identified 53.8% of all polyclonal samples in the study population. Indeed, inherent mutability in this MS has been described to produce excess diversity, which in turn is recommended to identify MOI [25].

We identified 101 haplotypes, of which 84 appeared only once in the population. Ninety percent of them were grouped in a single haplogroup (HG1), defined by identical alleles in at least 9/13 loci, indicating a high degree of relatedness among

parasites across the communities. These results support the view that despite a high level of polyclonality, inbreeding among highly related haplotypes maintains LD.

The adjusted genetic differentiation was low between communities 1, 2, and 3 ($F_{ST} < 0.05$) and moderate when community 4 was included ($F_{ST} = 0.15\text{--}0.18$), indicating limited geographical boundaries between neighboring communities 1-3 but higher differentiation with the community across the river. In concordance with the F_{ST} values, the STRUCTURE analysis detected two main parasite populations. Two clusters of haplotypes, with a high proportion of mixture haplotypes (60.4%) were observed in all four communities. The fact that a majority of haplotypes found in community 4 belonged to cluster 2, which was the minor cluster in the other 3 communities, supports a certain degree of differentiation between communities 1-3 and 4. Moderate population differentiation between these communities can be explained by geographical proximity and socioeconomic relationships between the communities' inhabitants as previously described [3]. Inhabitants of community 1-3 (located at one side of the river) belong to the Cadong ethnic group and therefore share some degree of kinship, facilitating social exchange. Conversely, community 4, whose inhabitants belong to the M'ong ethnicity, is located at the other side of the river with limited access during the rainy season.

Malaria incidence in the Quang Nam province has dropped by 78.0% over the last decade thanks to the implementation of efficient control strategies [1, 17, 57]. At the time of the study, malaria prevalence in the study area was 7.8% as assessed by light

microscopy and 23.6% as estimated by PCR [3]. Therefore, the moderate-to-high levels of genetic diversity detected, together with the high polyclonality and low population structure are consistent with an epidemiological context of transition from moderate to low endemicity [58-59].

Future studies aiming at identifying changes in genetic diversity and population structure to support the development or improvement of control and elimination interventions should include isolates collected at several time points from all areas where malaria is prevalent (or has been recently eliminated). Ideally, a molecular surveillance system should be implemented within the existing network of sentinel sites for drug resistance across the country to support evaluation of interventions and improve response strategies at the provincial level.

Parasite populations with strong LD and the presence of gene flow could fuel the spread of resistant parasites in the event of the emergence of drug resistance, threatening current treatment efforts and achievements towards malaria elimination in Central Vietnam. Temporal analysis to investigate haplotype persistence and the risk of clonal expansion is urgently needed in order to inform decision makers.

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References

1. National Institute of Malariology, Parasitology and Entomology: Annual Report of the National Malaria Control Program in Vietnam 2014, Hanoi, Vietnam. National Institute of Malariology, Parasitology and Entomology. Internal report 2015.
2. TT Duong, ND Thang: Malaria epidemiological stratification in Vietnam 2014. National report in Malariology, Parasitology and Entomology, National Institute of Malariology, Parasitology and Entomology Hanoi, Vietnam 11:21, Medical Publisher INSB 978-604-66-0950-6. Internal report 2015.
3. Pham VT, Nguyen VH, Nguyen VV, Malderen CV, Obsomer V, Rosanas-Urgell A, Grietens KP, Bancone G, Chowwiwat N, Tran TD, Nguyen XX, D'Alessandro U, Speybroeck N, Erhart A: Epidemiology of forest malaria in Central Vietnam: the hidden parasite reservoir. *Malar J* 2015, *14*:86.
4. Erhart A, Ngo DT, Phan VK, Ta TT, Van Overmeir C, Speybroeck N, Obsomer V, Le XH, Le KT, Coosemans M, D'Alessandro U: Epidemiology of forest malaria in central Vietnam: a large scale cross-sectional survey. *Malar J* 2005, *4*:58.
5. Trung HD, Van Bortel W, Sochantha T, Keokenchanh K, Quang NT, Cong LD, Cooseman M: Malaria transmission and major malaria vector in different geographical areas of southeast Asia. *Trop Med Int Health* 2004, *9*:230- 237.
6. National Institute of Malariology, Parasitology and Entomology: National strategy for malaria control and elimination for the period of 2011-2020 and orienting to 2030. Government of socialist republic Vietnam, (http://www.chinhphu.vn/portal/page/portal/chinhphu/noidungchienluocphat trienkinhtexahoi?piref135_16002_135_15999_15999.strutsAction=ViewDetailAction.do&piref135_16002_135_15999_15999.docid=1160&piref135_16002_135_15999_15999.substract=). Internal report 2011.
7. White NJ: Determinant of relapse periodicity in *Plasmodium vivax* malaria. *Malar J* 2011, *10*:297.

8. Mueller I, Galinsky MR, Baird JK, Carlton MJ, Kochar DK, Alonson PL, Adel Portillo H: Key gap in the knowledge of *Plasmodium vivax*: a neglected human malaria parasite. *Lancet Infect Dis* 2009, *9*:555-66.
9. Nguyen HV, Van den Eede P, Van Overmeir C, Thang ND, Hung Le X, D'Alessandro U, Erhart A: Marked age-dependent prevalence of symptomatic and patent infections and complexity of distribution of human *Plasmodium* species in central Vietnam. *Am J Trop Med Hyg* 2012, *87*:989-995.
10. Imwong M, Snounou G, Pukrittayakamee S, Tanomsing N, Kim JR, Nandy A, Guthmann JP, Nosten F, Carlton J, Looareesuwan S, Nair S, Sudimack D, Day NP, Anderson TJ, White NJ: Relapses of *Plasmodium vivax* infection usually result from activation of heterologous hypnozoites. *J Infect Dis* 2007, *195*:927-933.
11. World Health Organization (WHO): *Plasmodium vivax* control and Elimination: development of Global Strategy and Investment Case. Malaria policy Committee Meeting 11-13 September 2012, WHO HQ session 8 2012.
12. Thanh PV, Hong NV, NV Van, Melva L , Baird K, NX Xa, LX Hung, TT Duong, Rosanas-Urgell A, Speybroeck N, D'Alessandro U, Erhart A: Confirmed *Plasmodium vivax* resistance to chloroquine in Central Vietnam. *Antimicrob. Agents Chemother* 2015, *00791-15*
13. Van den Eede P, Erhart A, Van der Auwera G, Van Overmeir C, Thang ND, Hung le X, Anne J, D'Alessandro U: High complexity of *Plasmodium vivax* infections in symptomatic patients from a rural community in central Vietnam detected by microsatellite genotyping. *Am J Trop Med Hyg* 2010, *82*:223-227.
14. 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.
15. Christopher D, Veronica ES, Peter V DE, Dionica G, Angle R, Emmanuel N A, Hugo R, Alejandro L, Jean-Pierre V G, Annette E, Umberto D: Population structure and

- spatio-temporal transmission dynamics of *Plasmodium vivax* after radical cure treatment in a rural village of the Peruvian Amazon. *Malaria J* 2014,13:8.
16. Orjuela- Sanchez P, Sá JM, Brandi MC, Rodrigues PT, Bastos MS, Amaratunga C, Duong S, Fairhurst RM, Ferreira MU: Higher microsatellite in *Plasmodium vivax* than in sympatric *Plasmodium falciparum* population in Pursat western Cambodia. *Exp Parasitol* 2013, 134(3):318-326.
 17. Phuc BQ, ND Manh, Binh NTH, Ngoc NTH, Tan HV, Hanh NV, Hanh TV, Dao LD, Tinh TT: PCR investigation of malaria parasite structure in Nam Tra My District, Quang Nam province. National report in Malariology, Parasitology and Entomology, Hanoi, Vietnam 162:171. National Institute of Malariology, Parasitology and Entomology. 2011.
 18. Quang Nam provincial Malaria Station: Annual report on malaria entomological collections in Quang Nam province, Internal Report. Quang Nam provincial Malaria Station Tam Ky, Quang Nam, Vietnam. Internal report 2009 - 2013
 19. World Health Organization. Guidelines for the treatment of malaria. Second Edition, Geneva, Switzerland. [<http://apps.who.int/medicinedocs/en/d/Js19105en/>]. 2010.
 20. Rubio JM, Post RJ, van Leeuwen WM, Henry MC, Lindergard G, Hommel M: Alternative polymerase chain reaction method to identify *Plasmodium* species in human blood samples: the semi-nested multiplex malaria PCR (SnM-PCR). *Trans R Soc Trop Med Hyg* 2002, 96: S199--S204.
 21. Karunaweera ND, Ferreira MU, Hartl DL, Wirth DF: Fourteen polymorphic microsatellite DNA markers for the human malaria parasite *Plasmodium vivax*. *Molecular Ecology Notes* 2007, 7:172-175.
 22. Anderson TJC, Su X-Z, Bockarie M, Lagog M, Day KP: Twelve microsatellite markers for characterization of *Plasmodium falciparum* from finger prick blood samples. *Parasitology* 2009, 119:113-25.

23. Ferreira MU, Karunaweera ND, da Silva-Nunes M, da Silva NS, Wirth DF, Hartl DL: Population structure and transmission dynamics of *Plasmodium vivax* in rural Amazonia. *J Infect Dis* 2007, *195*: 1218–1226.
24. Koepfli C, Timinao L, Antao T, Barry AE, Siba P, Mueller Ivo, Felger I: A Large Reservoir and Little Population Structure in the South Pacific. *PLoS One* 2013, *8*: e66041.
25. Sutton P.L: A call to arms: on refining *Plasmodium vivax* microsatellite marker panel for comparing global diversity. *Malar J* 2013, *12*:447.
26. Peakall R, Smouse PE: GenAlEx 6.5 genetic analysis in Excel. Population genetic software for teaching and research –an update. *Bioinformatics* 10.1093. 2013.
27. Gunawardena S, Karunaweera ND, Ferreira MU, Phonr –Kyaw M, Pollack RJ, Alifrangis M, Rajakaruna RS, Konaradsen F, Amerasinghe PH, Schousboe ML, Galappaththy GN, Abeyasinghe RR, Hartl DL, Wirth DF: Geographic structure of *Plasmodium vivax*: microsatellite analysis of parasite populations from Sri Lanka, Myanmar and Ethiopia. *Am J Trop Med Hyg* 2010, *82*:235 -242.
28. Goudet J: FSTAT version 1.2: a computer program to calculate F-Statistic. *J Hered* 1995, *86*(6); 485-486.
29. Barry AE, Schultz L, Senn N, Nake J, Kiniboro B, Siba PM, Mueller I, Reeder J: High levels of genetic diversity of *Plasmodium falciparum* populations in Papua New Guinea despite variable infection prevalence. *Am J Trop Med Hyg* 2013, *88*: 718-725.
30. Haubold B, Hudson RR: LIAN 3.0: detecting linkage disequilibrium in multilocus data. *Bioinformatics* 2000, *16*:847-848.
31. Smith JM, Smith NH, O'Rourke M, Spratt BG: How clonal are bacteria? *Proc Natl Acad Sci USA* 1993, *90*:4384-4388.
32. Gout . J : FSTAT. <http://www2.unil.ch/popgen/softwares/fstat.htm>. Last access 15th July 2015.

33. Meirmans. P Recode Data v.0.1 (http://www.bentleydrummer.nl/software/recodeData_manual.pdf). Last access 15th July 2015.
34. Evanno G, Regnaut S, Goudet J: Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Mol Ecol* 2005, 14:2611-2620.
35. Jakobsson M, Rosenberg NA: CLUMPP: a cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. *Bioinformatics* 2007, 123 (14): 1801-6.
36. Noah A. Rosenberg NA: DISTRUCT: a program for the graphical display of population structure. *Molecular Ecology Note* 2004, 4: 137 -138.
37. Meirman PG, Van Tienderen PH: GENOTYPE and GENODIVE: two programs for the analysis of genetic diversity of asexual organisms. *Molecular Ecology Notes* 2004, 4: 792-794.
38. Dent A Earl, Bridgett M vonHoldt: Structure harvester: a website and program for visualizing STRUCTURE out put and emplimenting Evano method. *Conversation Genet Resour* 2014, 4: 359-361.
39. Evano G, Regnaut S, Goudet J: Detecting number of clusters of individual using the software STRUCTURE: a simulation study. *Mol Ecol* 2005, 14:2611-2620.
40. Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG: eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. *J Bacteriol* 2004, 186:1518-1530.
41. Fansisco AP, Vaz C, Monterio PT, Cristino JM, Ramirez M, Carrrio JA: Phyloviz: Phylogentic inference and data visualization for sequence base typing methods. *BMC Bioinformatics* 2012, 13- 87.
42. Gluikart G, Allendorf FM, Cornuet JM, Sherwin WB: Distortion of allele frequency distributions provides a test for recent population bottlenecks. *J Heredity* 1998, 89: 238-247.

43. Batista CL, Barbosa S, Da Silva Bastos M, Viana SA, Ferreira MU: Genetic diversity of *Plasmodium vivax* over time and space: a community –based study in rural Amazonia. *Parasitology* 2015, *142*:374- 384.
44. Orjuela-Sanchez P, DaSilva NS, da Silva- Nunes M, Ferreira MU: Recurrent parasitemias and population dynamic of *Plasmodium vivax* polymorphisms in rural Amazonia. *Am J Trop Med Hyg* 2009, *81*:961-968.
45. Iwagami M, Fukumoto M, Hwang S, Kim S, Kho W, Kano S: Population structure and transmission dynamic of *Plasmodium vivax* in the republic of Korea based on microsatellite DNA analysis. *PloS One* 2012, *6*:e1592.
46. Gunawardena S, Ferreira MU, Kapilananda GM, Wirth DF, Karunaweera ND: The Sri Lankan paradox: high genetic diversity in *Plasmodium vivax* populations despite decreasing level of malaria transmission. *Parasitology* 2014, *141*:880-890.
47. Arnott A, Barry AE, Reeder JC: Understanding the population genetic of *Plasmodium vivax* is essential for malaria control and elimination. *Malar J* 2012, *11*:14.
48. Imwong M, Nair S, Pukrittayakamee S, Sudimack D, Williams JT, Mayxay M, Newton PN, Kim JR, Nandy A, Osorio L, Carlton JM, White NJ, Day NP, Anderson TJ: Contracting genetic structure in *Plasmodium vivax* population from Asia and South America. *Int J Parasitol* 2007, *37*: 1013- 1022.
49. Karunaweera ND, Ferreira MU, Munasinghe A, Barnwell JW, Collins WE, King CL, Kawamoto F, Hartl DL, Wirth DF: Extensive microsatellite diversity in the human malaria parasite *Plasmodium vivax*. *Gene* 2008, *410*: 105 -112.
50. http://www.as.wvu.edu/~kgarbutt/QuantGen/Gen535_2_2004/Linkage_diseq_uilibrium.htm, Last access on 17th July, 2015.
51. Mzilahowa T, McCall PJ, Hastings IM: “Sexual” population structure and genetic of the malaria agent *P. falciparum*. *PloS One* 2007, *2*(7):e613.
52. Chenet SM, Schneider KA, Villgeas L, Escalante AA: Local population structure of *Plasmodium*: impact on malaria control and elimination. *Malar J* 2012, *11*:412.

53. Obaldia N, Barol NK, Calzada JE, Santamiaria AM, Daniel R, Wong W, Chang H, Hamilton EJ, Arevalo-Herrera M, Herrera S, Wirth DF, Hartl DL, Marti M, Wolkman SK: Clonal outbreak of *Plasmodium falciparum* in eastern Panama. *J Infect Dis* 2015, *211* (7):1087-1096.
54. Ferreira MU, Karunaweera ND, Silva- Nunes Md, Silva MSd, With FD, Hartl DL: Population structure and transmission dynamics of *Plasmodium vivax* in Rural Amazonia. *J Infect Dis* 2007, *195*:1218-26.
55. Levinson G: Slipped – Strand mispairing: a major mechanism for DNA sequence evolution. *Mol Biol Evol* 1987, *4* :203 -21.
56. Pearson CE, Nichol Edamura K, Cleary JD: Repeat instability: mechanisms of dynamic mutations. *Nat Rev Genet* 2005, *6*:729-742.
57. NV Van, NK Huy, NT Nhan, BQ Phuc: Some epidemiological aspect of malaria confirmed cases at Nam Tra My district, Quang Nam province in 2013. National report in Malariology, Parasitology and Entomology, Hanoi, Vietnam p22-30. National Institute of Malariology, Parasitology and Entomology. Internal report 2015.
58. Hay SI, Guerra CA, Gething PW, Patil AP, Tatem AJ, Noor AM, Kabaria CW, Manh BH, Elyazar IRF, Bookrt S, Smith DL, Moyeed RA: A world malaria map: *Plasmodium falciparum* endemicity in 2007. *PloS One* 2009, *6*:e1000048.
59. Erhart E, ND Thang, NQ Hung, LVToi, LX Hung, TQ Tuy, LD Cong, Speybroeck N, Coosemans M, D’Alessandro U: Forest malaria in Vietnam: A challenge for control. *Am J Trop Med Hyg* 2004, *70*:110-118.

Chapter 7

Delayed parasite clearance after treatment with dihydroartemisinin–piperaquine in *Plasmodium falciparum* malaria patients in central Vietnam

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Abstract

Reduced susceptibility of *Plasmodium falciparum* towards artemisinin derivatives has been reported from the Thai-Cambodian border and from the Thai-Myanmar border. Following increasing reports from central Vietnam of delayed parasite clearance after treatment with dihydroartemisinin-piperaquine (DHA-PPQ), the current first-line treatment, we carried out a study on the efficacy of this treatment.

Between September 2012 and February 2013, we conducted a 42-day *in vivo* and *in vitro* efficacy study in Quang Nam province. Treatment was directly observed and blood samples collected twice daily until parasite clearance. In addition, genotyping, quantitative PCR (qPCR), and *in vitro* sensitivity testing of isolates were performed. The primary endpoints were parasite clearance rate and time. The secondary endpoints included PCR-corrected and uncorrected cure rates, qPCR clearance profiles, *in vitro* sensitivity results (for chloroquine, Dihydroartemisinin, piperaquine) and genotyping for mutations in the Kelch 13 Propeller domain.

Out of 672 screened patients, 95 were recruited and 89 available for primary endpoint analyses. The mean parasite clearance time (PCT) was 61.7h (interquartile range [IQR]: 47.6 to 83.2h) and the median parasite clearance rate had a slope half-life of 6.2h (IQR: 4.4 to 7.5h). The PCR corrected efficacy rates were estimated at 100% at day 28 and 97.7% (95% confidence interval, 91.2% to 99.4%) at day 42. At day 3, the *P. falciparum* prevalence by qPCR was 2.5 times higher than by microscopy. The 50% inhibitory concentrations (IC_{50S}) of isolates with delayed clearance time (≥ 72 hours) were

significantly higher than those with normal clearance time in all three drugs. Delayed parasite clearance (PCT \geq 72h) was significantly higher among day 0 samples carrying the 543 mutant allele (47.8%) than those carrying the wild type allele (1.8%; $p=0.048$).

In Central Vietnam, the efficacy of DHA-PPQ is still satisfactory, but the parasite clearance time and rate are indicative of emerging artemisinin resistance. (This study has been registered at ClinicalTrials.gov under registration no. NCT01775592).

Introduction

Reduced susceptibility of *P. falciparum* towards artemisinin derivatives has been reported from the Thai-Cambodian [1-5] and more recently, from the Thai-Myanmar border [6, 7]. While exploring potential molecular markers for artemisinin resistance, delayed parasite clearance has been suggested as a proxy for resistance and was used to define adequate responses [8].

Vietnam has been using artemisinin derivatives for the treatment of malaria for more than twenty years [9]. An early Chinese version of artemisinin combination therapy (ACT), containing dihydroartemisinin (DHA) and piperaquine (PPQ) together with primaquine and trimethoprim, was produced and marketed in Vietnam as CV8, and was introduced in the National treatment guidelines in 2000 [10]. Since 2007, DHA-PPQ has been used as first line treatment for uncomplicated *P. falciparum* malaria [11].

Monitoring data on drug resistance collected from 1998 to 2009 in the southern province of Binh Phuoc in Vietnam, bordering Cambodia, showed stable sensitivity of *P. falciparum* to artemisinins, both *in vivo* and *in vitro* [12]. However, in 2010 and 2011, in the same area, the parasite clearance time was slower than in previous years [13]. Even though artemisinin combination therapies are still efficacious in treating malaria patients, prolonged clearance rates may indicate reduced susceptibility. During a recent study carried out by our research group in Quang Nam province, several *P. falciparum* malaria patients showed delayed clearance after supervised treatment

with DHA-PPQ. Moreover, the provincial malaria station reported an increasing number of cases with delayed parasite clearance since 2010. Therefore, the National Institute for Malaria, Parasitology and Entomology (NIMPE) in collaboration with the Institute of Tropical Medicine in Antwerp (ITM), Belgium, set up a study to assess the treatment response to DHA-PPQ in the same study site in Quang Nam province (registered at ClinicalTrials.gov under registration no. NCT01775592).

Material and Methods

Study site and participants

The study was conducted between September 9th, 2012 and February 1st, 2013 at the health center of Tra Leng commune, situated in North Tra My district, Quang Nam Province, central Vietnam. Patients attending the health center with suspected clinical malaria were screened for *P. falciparum* infection. Inclusion criteria were as follows: age >6 months, body temperature (axillary) $\geq 37.5^{\circ}\text{C}$ and/or history of fever in the previous 24 hours, *P. falciparum* mono-infection with parasite density between 500 and 100,000/ μl by light microscopy (LM), and provision of written informed consent to participate in the trial. For patients aged <18 years, informed consent was obtained from the parents/guardians. Pregnant (as determined by the β - human chorionic gonadotropin test) or lactating women were excluded. Similarly, patients with concomitant acute or chronic severe illness, severe malnutrition, severe danger signs or signs of severe malaria were excluded. In addition, for *in vitro* testing, we excluded patients who had received quinine, artemisinin or artemisinin-derivatives within 7

days, 4-aminoquinolines within 14 days, pyrimethamine and/or sulfonamides within 28 days, or mefloquine within 56 days before screening.

Study design

The study was designed as a 42-day follow-up study to assess the efficacy of DHA-PPQ in treating clinical *P. falciparum* malaria. Patients either stayed at the health facility for the duration of the treatment and until parasite clearance or were asked to return every 12 h for blood sampling and directly observed treatment. Patients were then asked to return for follow-up visits at days 7, 14, 21, 28, 35 and 42 or whenever they had signs and symptoms consistent with malaria. All patients received DHA-PPQ, according to the national guidelines based on an age-dependent dosing scheme[14], corresponding in our study population to approximately 4mg/kg DHA plus 32mg /kg PPQ on day 0 and 2mg/kg/day DHA plus 17mg/kg/day PPQ on days 1 and 2. The DHA-PPQ is produced in Vietnam and provided by the National Pharmaceutical Joint Stock Company I, Vietnam (lot No: 11007, registration no. VD-12944-10).

Clinical procedures

For each patient, a general physical examination was performed at enrollment and any other visit. Adverse drug reactions and concomitant medication were recorded at every visit. A finger prick blood sample was taken every 12 h (± 2 h) until two consecutive blood slides were found negative for *P.falciparum*, and then weekly from days 7 to 42 and at any unscheduled visit. From each sampling, thick and thin blood films were prepared, and approximately 50 μ l of blood were collected on filter paper (Whatman grade 3) for

later PCR analysis. Each filter paper was dried and individually stored in a plastic bag containing silica gel. At days 0, 14, 28 and 42, additional blood sample was taken to measure hemoglobin concentration using the HaemoCue method. Additionally, at enrolment, 200µl of blood was taken into a heparinized capillary tube and rapidly transferred into culture medium (RPMI 1640 LPLF liquid medium) for *in vitro* testing.

Laboratory procedures

Microscopy

Thick and thin films were stained and the parasite density determined by counting the number of asexual parasites per 200 white blood cells (WBCs) assuming a WBC count of 8000/µl. A slide was considered negative if no asexual parasites were found after counting 1,000 WBCs. Systematic double reading by two independent readers was done for all slides taken before treatment and for the first two negative slides. Discrepant results were re-read by a third senior technician. Discrepant in the results between the two microscopists was defined as positive/negative slide, a differences in species diagnosis or as >25% difference in parasite density. Each final parasite density was computed by averaging the two most concordant counts. For each positive/negative slides, the third reading was taken as the final result. External quality control on 10% of randomly selected slides was done at the ITM, Belgium.

Genotyping of recurrent infections

Filter papers with dried blood samples were punched and one circle of 5mm in diameter was used for DNA extraction with a QIAamp DNA Blood macro kit (Qiagen) following manufacturer's recommendations. Extracted DNA was eluted in 50µl water and used for the genotyping of recurrent *P. falciparum* infections following WHO recommendations[15] by characterizing the length polymorphism of the *Msp1*, *Msp2* and *Glurp* genes in samples collected at day 0 and day of recurrent parasitaemia was found. Recrudescence was determined when, for each marker (*Msp1*, *Msp2*, *Glurp*) at least one identical-length polymorphism was found between samples collected on day 0 and on the day of recurrent infection. A new infection was defined when for at least one marker, the length polymorphisms were different between the sample collected on day 0 and that collected on the day of recurrent infection.

Genotyping K13

Alleles of four Kelch 13 (K13) propeller domain polymorphisms (Y493H, R539T, I543T, and C580Y) associated with delayed clearance [16] were determined by TaqMan allelic discrimination and sequencing. Amplification was done with 300nM of the forward and reverse primers, 200nM each of the allele-specific probes and at least 5ng of DNA on the Bio-Rad CFX96 real-time thermocycler set to detect fluorescent emissions for 6-carboxyfluorescein (6FAM) (mutant) and hexachloro -6- carboxyfluorescein (HEX) (wild type). Allelic discrimination analysis was performed with the Bio-Rad CFX manager with parameters set to subtract background and correct for fluorescent drift

prior to clustering of wild-type or mutant amplicons. For a subset of samples, TaqMan determined K13 single-nucleotide polymorphisms (SNPs) were confirmed by sequencing of amplicons by BigDye3.1 terminator chemistry (Life Technologies) of a 400bp K13 fragment with the primers K13-493 (Fwd-GCTGGCGTATGTGTACACCTATG) and k13-580 (Rev-ATCTCTCACCATTAGTCCACCAAT). Sequence traces obtained from a 3130xl genetic analyser were assembled and analysed against the 3D7 reference sequence in Seqscape version 2.5 software.

Quantitative PCR

Quantitative real time PCR was done on all day 0 samples, on the first of the two samples collected at day 1 and 2 and then until clearance of infection as determined by microscopy (days 1 to 5), and on all follow up samples from day 7 to day 42. DNA extraction from filter papers was performed as described above. Duplex quantitative PCR (qPCR) for the detection and quantification of *P. falciparum* and *P. vivax* was performed on day 0 samples as described elsewhere [17]. Single *P. falciparum* qPCRs were used from day 1 until day 42. Quantification was performed by converting the threshold cycle (C_T) into 18S gene copy number by using standard curves constructed using two positive control plasmids with the respective 18S amplicons inserted. A standard curve for *P. falciparum* was included in every qPCR run with a 10-fold dilution starting from 10^6 copies/ μ l of *P. falciparum*. The lower limit of the quantification was 1 parasite/ μ l and the precision of the qPCR assay was 1-2 parasite/ μ l.

In vitro drug sensitivity assay

Samples collected on day 0 were tested immediately after collection from the enrolled patients using WHO MARK III method [18]. *In vitro* micro-test plates were freshly coated by the study team and kept at 4°C with Piperaquine (PPQ) (range, 12.5 – 800 nmol/liter blood medium mixture [BMM]), Dihydroartemisinin (DHA) (range, 0.25 – 16 nmol/liter BMM) and Chloroquine (CQ) (range, 20 – 1,280 nmol/liter BMM) following the procedure described by WHO [18]. The final composition of 1ml BMM consisted of 100µl; unwashed whole blood and 0.9ml RPMI1640 liquid medium. The final volume in each well was 75µl. All plates were incubated in candle jars.

The tests were considered valid when $\geq 10\%$ of the parasites in the control well had reached schizont stage, defined as parasites with three or more nuclei, within 24-36 h. The number of schizonts per 200 parasites was counted and used as a measure of maturation inhibition. Slides were read by three independent readers. The mean of the two closest readings was used as final result. External quality control was done on 10% of randomly chosen slides derived from the *in vitro* assay by the Shoklo Malaria Unit (SMRU) in Mae Sot, Thailand.

Study Endpoints

The primary endpoints were parasite clearance rate and parasite clearance time. Parasite clearance rate was defined according to the WorldWide Antimalarial Resistance Network (WWARN) guidelines [19]. The parasite clearance time (PCT) was

defined as the time elapsed between the patient's first dose and the time of the first negative blood slide.

Secondary study endpoints were treatment failure (PCR-adjusted and unadjusted) at day 28 and day 42, fever clearance time, and the 50% inhibitory concentration (IC₅₀) of DHA, PPQ and CQ as determined by *in vitro* assay. Gametocyte carriage and transmission potential were measured by calculating gametocyte positivity and person-gametocyte-week (PGW) rates as previously described [20]. The PGW rate was defined as the total number of weeks with detectable gametocytes in all patients during the first 2-week follow up divided by the total number of follow-up weeks. Treatment failures were categorized as early (ETF) and late treatment failures (LTF), with the LTFs further categorized as late clinical (LCTF) or parasitological (LPTF) failures [21].

Sample size

Assuming that 5% of patients would still be parasitaemic at day 3, 90 patients would be able to estimate this with a 5% precision and a 10% security margin. The estimation assumes also that a maximum 20% of included patients would be lost to follow up.

Data analyses

Data were entered into Access database (Microsoft Office 2007), and all statistical analyses were performed using STATA 12 (StatCorp, USA). Descriptive statistics were computed and differences were compared using either a Chi-square test for

categorical variables, or the Mann-Whitney U test or the nonparametric equality-of-medians test as required for continuous variables. The online parasite clearance estimator (PCE) tool developed by the WWARN [19] was used to determine the parasite clearance rate. Treatment success was assessed by Kaplan-Meier analyses.

A sigmoid, 4-parameter concentration inhibition model was applied to generate IC_{50} estimates using the *In Vitro* Analysis and Reporting Tool (IVART)[22]. Only results that met the core criteria as defined by IVART were included in the analyses. The mean IC_{50} s from patients with normal parasite clearance rate those and those with delayed parasite clearance were compared using Mann-Whitney U test. The correlation between IC_{50} and PCT was assessed using the partial correlation method.

Quantitative PCR parasitemia (expressed as copy numbers/ μ l) were analyzed to describe the different clearance profiles observed. The associations between each qPCR clearance profile and other variables were assessed, and a multivariate logistic regression model was used to determine the independent association of the different qPCR profiles with the day 3 parasite positivity as determined by LM. The prevalence of the four SNPs was estimated at day 0 and the risk of delayed parasite clearance (PCT \geq 72h) was compared between the day 0 samples carrying the mutant allele and those carrying the wild – type allele.

Ethical considerations

The study was approved by the Institutional Review Board of the ITM (Antwerp, Belgium), the ethical committee of University Hospital (Antwerp) and the ethical committee of the NIMPE (Hanoi, Vietnam).

Results

Trial profile and baseline characteristics

Of the 672 patients screened, 111 (16.5%) were infected with *P. falciparum*, 16 (14.4%) of whom did not fulfill the entry criteria, resulting in 95 (85.6%) patients recruited (Figure 1).

Eighty-nine (93.7%) patients completed the 3 day course of DHA-PPQ and were available for the primary endpoint analyses, and 88 (92.6%) patients completed the 42-day follow-up. Males (55.8%) slightly outnumbered females, with age ranging from 1 to 60 years (Table 1). For most (89.5%) patients, onset of fever occurred within previous 72h and 81% had measurable fever at time of recruitment. Gametocytes were found at very low densities in 18% of the patients.

The study drug was well tolerated; one patient vomited the first dose within the first half an hour and was retreated with a full dose. No serious adverse drug reaction was recorded.

Primary endpoints

Parasite clearance rate was assessed in 87.6% (78/89) patients; the median slope half-life was estimated at 6.2 h (interquartile range [IQR], 4.4 to 7.5h).

The parasite clearance time ranged from 14.1 to 120.7 h, with a median of 61.7 hours (IQR, 47.6 to 83.2) (Table2). Almost one-third of the patients were still positive for *P. falciparum* trophozoites at day 3 and all infections were cleared by day 5 (Table 2).

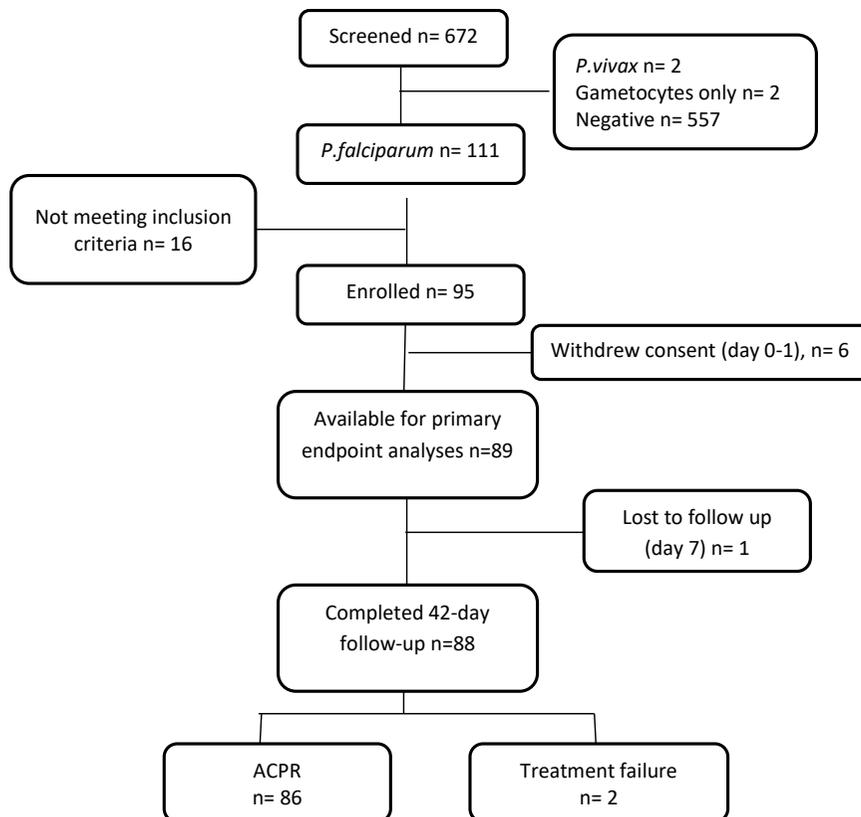


Figure 1. Follow chart of patients in the study

ACPR, adequate clinical and parasite sitological response.

Table 1. Baseline characteristics of the study population at enrolment^a

Characteristics	Patient data
Male, n (%)	53 (55.8)
Age (median [IQR]) (yr)	13 (6-29)
Age group (n [%])	
1-5 yr	36(37.9)
6-15 yr	19 (20.0)
>15 yr	40 (42.1)
Weight (kg) by age group, Median [IQR]	
1-5 yr	14 (9.5-15)
6-15 yr	23 (20-24)
>15 yr	49 (44-53.5)
History of fever: n (%)	
≤3 days	85(89.5)
>3 days	10 (10.5)
Fever (axillary temp° ≥37.5°C) (n [%])	77 (81.1)
Body temperature among febrile patients (median [IQR]) (°C)	38.4 (37.8-39)
Trophozoites density (geometric mean [95%CI]) (per μl)	8,233 (6,047 - 11,2010)
Presence of gametocytes (n [%])	18 (18.9)
Gametocytes density (n=18) (geometric mean [95%CI]) (per μl)	6.2 (3.2-11.9)
Haemoglobin (mean ±SD) (g/dl)	12.2 ±2.4
Heart rate (median [IQR]) (per min)	87 (82-90)
Respiratory rate (median [IQR]) (per min)	28 (27-30)

^a n= 95

Table 2. Primary and secondary endpoints

Endpoint ^a	Value
Primary Endpoints	
<i>Parasite clearance rate (n=78)^b</i>	
Lag phase (n=5) median [range] (h)	11 (11-38)
Parasite clearance rate constant median [IQR] (per h)	0.11/h (0.09-0.16)
Slope half-life, median [IQR] (h)	6.2 (4.4-7.5)
<i>PCT (n=89) (h)</i>	
PCT (median [IQR])	61.7 (47.6-83.2)
PC ₅₀ , (median [IQR])	8.7 (5.4- 12.1)
<i>Positive parasitemia from day3 onwards (n=89) (n [%])</i>	
Day 3 first slide ^c	26 (29.2)
Day 3 second slide	17 (19.1)
Day 4 first slide	8 (9.0)
Day 4 second slide	2 (2.2)
Day 5 first slide	0 (0)
Secondary Endpoints	
<i>Treatment outcomes (n=89)</i>	
LPF (PCR corrected) (n)	2
<i>PCR Corrected Cure Rate (K-M^d)</i>	
At day 28 (n[%])	89 (100)
At day42 (n[n]) [95% CI]	87 (97.7) [91.2;9.44]
<i>FCT (n=72), median [IQR] (h)</i>	12.0 h (11.1-13.6)
Gametocyte carriage (person-weeks)	49.1/1,000

^a PCT, parasite clearance time. PC₅₀, 50% parasite clearance rate; PLF, late parasitological failure; FCT, fever clearance time.

^b As estimated by the PCE (WWARN) [19];

^c blood samples were taken every 12h until parasite clearance.

^d K-M=Kaplan- Meir.

Secondary endpoints

No ETF was observed; two patients had a recurrent infection, at day 38 and 42 (LPF), confirmed to be recrudescences by genotyping. Therefore, the PCR corrected adequate clinical and parasitological response was estimated at 100% at day 28 and 97.7% (95% confidence interval [CI]: 91.2% to 99.4%) on day 42. Among the 18 patients with gametocytes at day 0, eight (44.4%) still had gametocytes at day 7; none of the patients without gametocytes on day 0 had gametocytes in the course of the follow up. Gametocyte carriage was estimated at 49.1/1,000 person-gametocyte-weeks (Table 2).

K 13 results

A total of 83 samples collected at enrolment were examined for the four SNPs of the K13 propeller domain. The overall prevalence of the mutant allele was 80.7% (67/83) at positions 543, 1.3% (1/78) at position 493 and none at positions 539 and 580. Delayed parasite clearance ($PCT \geq 72h$) was significantly higher among day 0 samples carrying the 543 mutant allele (47.8%) as than those carrying the wild type allele (1.8%; $p=0.048$). Taking into account only samples in which the slope half-life was accurately estimated by the parasite clearance estimator, the difference was even more significant, that is, 53% versus 21%, ($p=0.04$). The sensitivity and specificity of the SNP543 at day 0 to identify delayed clearance ($PCT \geq 72h$) was therefore estimated at 91.4% (32/35) and 27.1% (13/48) respectively; the positive predictive value of the

mutation being only 47.8% (32/67) while the negative predictive value of the wild type allele was 81.3% (13/16).

In vitro results

In vitro sensitivity testing was successfully performed in 51 (53.7%) samples, 43 (82.3%) tested for CQ, 39 (76.5%) for DHA, and 48 (94.1%) for PPQ. The geometric mean IC₅₀s was 128.65nmol/liter for CQ, 1.07nmol/liter for DHA, and 94.83nmol/liter for PPQ. Patients with delayed parasite clearance had a significantly higher IC₅₀ than did those with normal clearance for all three drugs (Table 3).

Pairwise comparisons between the three drugs showed a significant positive correlation between the IC₅₀s of CQ and those of PPQ ($p=0.007$). No correlation was found between PCT and IC₅₀s values for DHA ($p=0.31$) while such correlation was significant for both PPQ ($p=0.05$) and CQ ($p=0.04$), even after controlling for parasite density at enrollment.

Table 3: IC₅₀s of CQ, DHA and PPQ overall and comparison by PCT^a

Drug	Overall IC ₅₀		Comparison by PCT			
			IC ₅₀ Geometric mean [95%CI] (nM) by PCT of:			
	n	IC ₅₀ Geometric mean [95% CI] (nM)	n	<72h	≥72h	P value ^b
CQ	43	128.65 (98.70-167.70)	41	90.92 (61.76-133.83)	166.83 (115.26- 241.47)	0.015
DHA	39	1.07 (0.78-1.46)	36	0.83 (0.51- 1.35)	1.60 (1.05- 2.45)	0.010
PPQ	48	94.83 (65.87- 136.53)	45	53.54 (31.18- 91.95)	149.37 (91.19- 244.66)	0.008

^a IC₅₀, 50% inhibitory concentration; CQ, chloroquine, DHA, dihydroartemisinin; PPQ, parasite clearance time.

^bMann-Whitney U test

qPCR results

A total of 88 patients were analyzed by qPCR assay. The prevalence and mean parasite density (copy numbers/μl) during the first week after treatment are shown in Figure 2.

Compared to microscopy, qPCR detected increasingly more infections over time, and the difference became significant at day 3 when the prevalence by PCR was 2.5 times higher than by LM (77.3% (68/88) versus 29.5% (26/88); risk ratio (RR) =2.61, 95%CI [1.86; 3.68]; *p*<0.001). When taking into account the infections (identified by LM) carrying only gametocytes at day 3 (n=4), the RR remained significant (RR=2.27; *p*<0.001), and this was also the case at day 7 (29.5% (26/88) versus 9.1% (8/88);

RR=3.25 [95% CI 1.56 to 6.78], $p<0.001$). Three main profiles of qPCR density until day 7 were identified: i) individuals who cleared infection by day 3 (group1); ii) those who cleared between day 4 and 6 (group2); and iii) those positive until day 7 (group 3). Associations between the three qPCR profiles and potential risk factors are shown in Table 4.

qPCR positivity at days 4 to 6 (group 2) was significantly associated with higher LM parasite density, higher PCT and temperature at enrollment, as compared to group 1 and 3. Nevertheless, the qPCR positivity at day 7 was associated with a higher prevalence of gametocytes at enrollment ($p=0.01$) and with gametocyte carriage ($p=0.003$). After adjusting for the effect of parasite density, fever and gametocytes at enrollment, the delayed parasite clearance by LM was strongly associated with the qPCR positivity at day 3 (adjusted odds ratio [OR], 9.55, [95%CI 1.79 to 50.93], $p=0.008$) but not at day 7 (adjust OR, 1.34, $p=0.76$) or during the weekly follow-up visits ($p=0.87$).

External quality control

External quality control for standard microscopy at ITM and for microscopy of *in vitro* slides at SMRU showed comparable results.

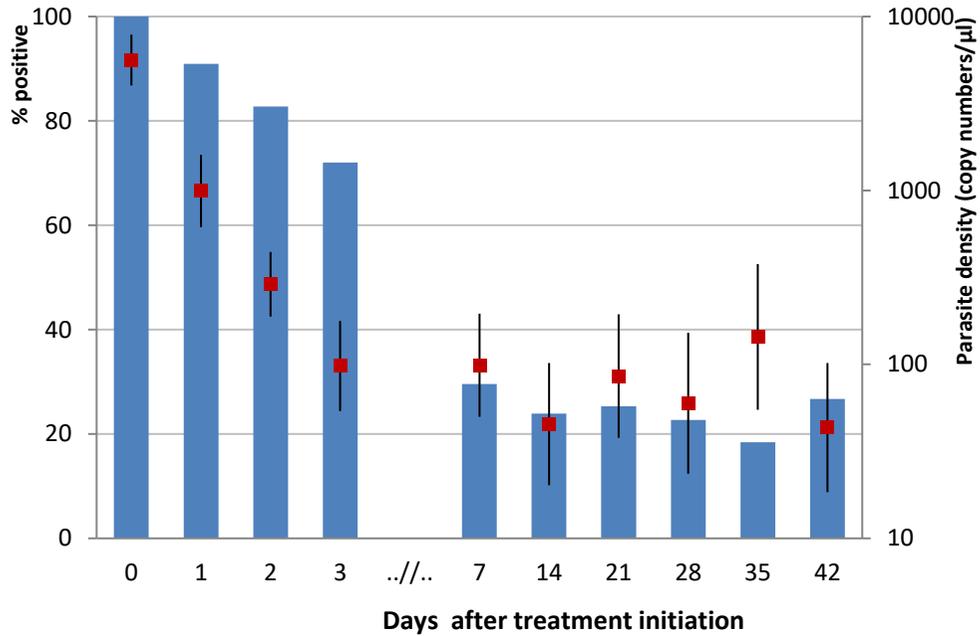


Figure 2. Parasite prevalence and mean density determined by quantitative PCR (qPCR) from day 0 to 3 then weekly from day 7 to 42.

Left y-axis, parasite prevalence determined by qPCR (% positive samples); right y-axis, mean parasite density expressed as the geometric mean of copy numbers/ μ l (red squares) with error bars showing the upper and lower limits of the 95% CIs.

Table 4. Characteristics of patients by qPCR profiles during the first week^a

Times and characteristics	Data for infection			p-value
	Cleared by day3 (n=18)	Cleared day 4-6 (n=44)	Not cleared by day 7 (n=26)	
<i>At day 0</i>				
Age (yr)	20 (5-32)	12 (5.5- 28)	11.5 (5-16)	0.26
Gender (% females)	55.6	45.5	38.5	0.5
Temperature (°C)	37.7 (37.0-38.3)	38.9 (38.0- 39.4)	37.8 (37.6- 38.5)	0.006
Hemoglobin (g/dl)	12.2 (9.8- 13.4)	13.3 (11.1-14.3)	11.3 (9.6-12.6)	0.17
Trophozoite density (LM) ^b	3,187 (1,533-6,622)	15,746 (10,816-22,922)	5,373 (3,002-9,620)	<0.05
Gametocyte prevalence (%)	22.2	9.1	38.5	0.010
IC ₅₀ DHA, (nmol/liter) ^c	0.44 (0.39- 2.36)	1.1 (0.72- 2.34)	0.58 (0.50- 2.73)	0.10
IC ₅₀ PPQ, (nmol/liter) ^d	101.6 (41.6-400.1)	101.1 (37.7- 391.1)	44.8 (21.0-149.9)	0.63
<i>During follow-up</i>				
PCT (h)	52.4 (34.9- 60.3)	82.5 (61.4- 96.5)	37.3 (24.1-62.3)	<0.001
Gametocyte carriage ^e	37.7	26.5	96.2	0.003
Pf infections by PCR after day 7 (%)	33.3	38.6	92.3	<0.001

^a Data are presented as median (IQR) unless otherwise specified.

^b Geometric mean of trophozoites density [95%CI];

^cTotal samples analyzed n=5, 19, and 19 for group 1, 2, and 3 respectively (p-value for group 2 vs group1 & 3);

^dTotal samples analyzed n=6, 28, 11, for group 1,2 and 3, respectively.

^egametocyte carriage per 1000person-weeks (group 1 and 2 were compared to group3; incidence rate ratio 0.31; 95%CI 0.13 to 0.72, p=0.003);

Discussion

Considering 30% of enrolled patients were still parasitaemic at day 3, this area of Central Vietnam fulfills the WHO definition of suspected artemisinin resistance [8].

Earlier studies from Southern Vietnam reported a 48-h PCT after 3 day DHA-PPQ treatment (DHA 2.4mg/kg/day, PPQ 19.2mg/kg/day) and a clearance half-life of 2.98 h [13]. This is considerably shorter than the PCT and clearance half-life time found in our study, in which we used the dosage recommended by the Vietnamese Ministry of Health [14]. As this dosing scheme is based on age, the risk of underdosage is high and could have resulted in a less-than-optimal treatment efficacy. However, when computing the average dosage according to the actual patients' bodyweight, this was 4.4mg/kg and 2.2mg/kg for DHA and 28.4mg/kg and 14.2mg/kg for PPQ on day 0 and days 1 and 2, respectively. In addition, there was no difference in dosage between patients with delayed clearance and others (data not shown). When considering that our dosing scheme resulted in a higher total dosage per patient than in a previous study carried out in Binh Phuoc Province [13], it is unlikely that under-dosage was the cause of the observed delayed parasite clearance.

The significantly higher IC₅₀s values for CQ, DHA and PPQ in isolates obtained from patients with delayed clearance provide an additional argument for a true increased tolerability of local parasite isolates. Moreover, the significant correlation between CQ and PPQ IC₅₀s found in our study might indicate the presence of cross-resistance between the two drugs. The absence of correlation between PCT and IC₅₀s of DHA

confirms earlier results from Cambodia [4, 5] and corroborates recent findings on the ability of *P. falciparum* artemisinin resistant strains to arrest their development at early ring stage [23].

Previous studies have suggested that SNPs in the propeller region of the Kelch 13 protein may be used as molecular markers for artemisinin resistance [24, 25] and recent field studies have confirmed an association between the mutation and slow clearance [26]. In our study, despite the high prevalence of the 543 mutation, infection with the mutant allele had a significantly longer parasite clearance time (PCT \geq 72h) than did wild-type infections. Most of patients with delayed parasite clearance carried the mutation, hence the high sensitivity, while specificity was low because of the mutation's high prevalence among all patients. Mutations in other positions may contribute to the observed delayed clearance. Sequencing of day 0 samples and of infection detected at day 3 is currently ongoing and may yield information on the importance of additional mutations.

As expected, more patients were positive by qPCR than by LM during the course of the follow up. PCR positivity at day 3 was significantly associated with delayed parasite clearance by LM and submicroscopic infections during the weekly follow-up were not associated with delayed clearance. We therefore hypothesize that this might represent circulating gametocytes as shown in other studies [27]. This is plausible when considering that patients positive by qPCR at day 7 were more likely to carry gametocytes before treatment and during the follow-up. Treatment with DHA-PPQ

has been associated with a higher production of gametocytes than that of other ACTs, though it is unclear whether these persisting gametocytes are still viable and infectious to the vector [28]. In Kenya, residual parasitaemia was associated with a 2-fold longer duration of gametocyte carriage in children [27]. Although ACTs have a moderate effect on gametocytemia, submicroscopic gametocyte carriage occurs frequently and is sufficient to affect post-treatment transmission in a setting in which malaria is highly endemic [29]. To what extent submicroscopic infections contribute to the transmission and spread of resistant parasite strains needs to be addressed. This was an observational study with only one treatment arm, not an ideal design to distinguish between resistance to artemisinin derivatives and the partner drug. A randomized trial comparing DHA-PPQ with artesunate monotherapy would have provided the opportunity to exclude the interference of the partner drug and to confirm artemisinin resistance in the study area. Plans for such a study are underway.

Correlating DHA *in vitro* and *in vivo* results is difficult since standard *ex-vivo* assays measure schizont maturation while artemisinin resistant parasites can block their development at the early ring stage. Ring-stage survival assays would have been more appropriate to measure ring stage survival under high DHA concentration [30] or similarly, a trophozoite maturation assay might have been more suitable to detect resistant parasites [31]. However, at the time the study was conducted, those tests were not yet available. Further comparison between *in vitro* results obtained in different laboratories has to be interpreted with caution.

In conclusion, our study shows that, though efficacy of DHA-PPQ is still satisfactory, one-third of the *P. falciparum* infections were not cleared at day 3, defining the study area as highly suspicious for artemisinin resistance. Analyses of potential mutations in the PF3D7_1343700 kelch propeller domain ('K13) propeller domain [16] are ongoing and will be reported separately. Though resistance has yet to be confirmed, our results prompted the National Malaria Control Program (NMCP) together with WHO to declare Quang Nam province as a Tier I area since May 2013 [32] as part of the Global Plan for Artemisinin Resistance Containment (GPARC). After similar reports from Binh Phuoc, Gia Lai and Dak Nong, this is the fourth province and the first one in a non-bordering area in Central Vietnam reporting suspected artemisinin resistance. These reports raise serious concerns on the emergence or spread of multidrug-resistant strains in the Great Mekong sub-region.

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References

1. Noedl H, Se Y, Schaecher K, Smith BL, Socheat D, Fukuda MM, Artemisinin Resistance in Cambodia 1 Study C: Evidence of artemisinin-resistant malaria in western Cambodia. *N Engl J Med* 2008, *359*:2619-2620.
2. Noedl H, Se Y, Sriwichai S, Schaecher K, Teja-Isavadharm P, Smith B, Rutvisuttinunt W, Bethell D, Surasri S, Fukuda MM, et al: Artemisinin resistance in Cambodia: a clinical trial designed to address an emerging problem in Southeast Asia. *Clin Infect Dis* 2010, *51*:e82-89.
3. Noedl H, Socheat D, Satimai W: Artemisinin-resistant malaria in Asia. *N Engl J Med* 2009, *361*:540-541.
4. Amaratunga C, Sreng S, Suon S, Phelps ES, Stepniewska K, Lim P, Zhou C, Mao S, Anderson JM, Lindegardh N, et al: Artemisinin-resistant *Plasmodium falciparum* in Pursat province, western Cambodia: a parasite clearance rate study. *Lancet Infect Dis* 2012, *12*:851-858.
5. Dondorp AM, Nosten F, Yi P, Das D, Phyo AP, Tarning J, Lwin KM, Arie F, Hanpithakpong W, Lee SJ, et al: Artemisinin resistance in *Plasmodium falciparum* malaria. *N Engl J Med* 2009, *361*:455-467.
6. Phyo AP, Nkhoma S, Stepniewska K, Ashley EA, Nair S, McGready R, ler Moo C, Al-Saai S, Dondorp AM, Lwin KM, et al: Emergence of artemisinin-resistant malaria on the western border of Thailand: a longitudinal study. *Lancet* 2012, *379*:1960-1966.
7. Parker D, Lerdprom R, Srisatjarak W, Yan G, Sattabongkot J, Wood J, Sirichaisinthop J, Cui L: Longitudinal in vitro surveillance of *Plasmodium falciparum* sensitivity to common anti-malarials in Thailand between 1994 and 2010. *Malar J* 2012, *11*:290.
8. WHO: Update on artemisinin resistance - April 2012. 2012.
9. Tran TH, Dolecek C, Pham PM, Nguyen TD, Nguyen TT, Le HT, Dong TH, Tran TT, Stepniewska K, White NJ, Farrar J: Dihydroartemisinin-piperaquine against

- multidrug-resistant *Plasmodium falciparum* malaria in Vietnam: randomised clinical trial. *Lancet* 2004, 363:18-22.
10. Giao PT, de Vries PJ, Hung le Q, Binh TQ, Nam NV, Kager PA: CV8, a new combination of dihydroartemisinin, piperazine, trimethoprim and primaquine, compared with atovaquone-proguanil against falciparum malaria in Vietnam. *Trop Med Int Health* 2004, 9:209-216.
 11. Ministry of Health NMCP: Guideline for malaria diagnosis and treatment in Vietnam 2007
 12. Thanh NV, Toan TQ, Cowman AF, Casey GJ, Phuc BQ, Tien NT, Hung NM, Biggs BA: Monitoring for *Plasmodium falciparum* drug resistance to artemisinin and artesunate in Binh Phuoc Province, Vietnam: 1998-2009. *Malar J* 2010, 9:181.
 13. Hien TT, Thuy-Nhien NT, Phu NH, Boni MF, Thanh NV, Nha-Ca NT, Thai le H, Thai CQ, Toi PV, Thuan PD, et al: In vivo susceptibility of *Plasmodium falciparum* to artesunate in Binh Phuoc Province, Vietnam. *Malar J* 2012, 11:355.
 14. Ministry of Health NMCP: Guidelines for malaria diagnosis and treatment in Vietnam. 2013.
 15. WHO: Methods and techniques for clinical trials on antimalaria drug efficacy: genotyping to identify parasite populations. 2008.
 16. Ariey F, Witkowski B, Amaratunga C, Beghain J, Langlois AC, Khim N, Kim S, Duru V, Bouchier C, Ma L, et al: A molecular marker of artemisinin-resistant *Plasmodium falciparum* malaria. *Nature* 2014, 505:50-55.
 17. Rosanas-Urgell A, Mueller D, Betuela I, Barnadas C, Iga J, Zimmerman PA, del Portillo HA, Siba P, Mueller I, Felger I: Comparison of diagnostic methods for the detection and quantification of the four sympatric *Plasmodium* species in field samples from Papua New Guinea. *Malar J* 2010, 9:361.
 18. WHO: Field application of in vitro assays for the sensitivity of human malaria parasites to antimalaria drug. ISBN 92 4 159515 9, Geneva 2007.

19. Flegg JA, Guerin PJ, White NJ, Stepniewska K: Standardizing the measurement of parasite clearance in falciparum malaria: the parasite clearance estimator. *Malar J* 2011, *10*:339.
20. Price RN, Nosten F, Luxemburger C, ter Kuile FO, Paiphun L, Chongsuphajaisiddhi T, White NJ: Effects of artemisinin derivatives on malaria transmissibility. *Lancet* 1996, *347*:1654-1658.
21. WHO: Methods for surveillance of antimalaria drugs efficacy. *Last accessed 05032013 Available from: <http://www.who.int/malaria/publications/atoz/9789241597531/en/> 2009*
22. Woodrow CJ, Dahlstrom S, Cooksey R, Flegg JA, Le Nagard H, Mentre F, Murillo C, Menard D, Nosten F, Sriprawat K, et al: High-throughput analysis of antimalarial susceptibility data by the WorldWide Antimalarial Resistance Network (WWARN) in vitro analysis and reporting tool. *Antimicrob Agents Chemother* 2013, *57*:3121-3130.
23. Witkowski B, Khim N, Chim P, Kim S, Ke S, Kloeung N, Chy S, Duong S, Leang R, Ringwald P, et al: Reduced artemisinin susceptibility of *Plasmodium falciparum* ring stages in western Cambodia. *Antimicrob Agents Chemother* 2013, *57*:914-923.
24. Cheeseman IH, Miller BA, Nair S, Nkhoma S, Tan A, Tan JC, Al Saai S, Phyo AP, Moo CL, Lwin KM, et al: A major genome region underlying artemisinin resistance in malaria. *Science* 2012, *336*:79-82.
25. S T-H: Genetic loci associated with delayed clearance of *Plasmodium falciparum* following artemisinin treatment in Southeast Asia. *Proceedings of the National Academy of Sciences of the United States of America*, 2013, *110*(1): p 240-5 2013.
26. Ashley EA, Dhorda M, Fairhurst RM, Amaratunga C, Lim P, Suon S, Sreng S, Anderson JM, Mao S, Sam B, et al: Spread of artemisinin resistance in *Plasmodium falciparum* malaria. *N Engl J Med* 2014, *371*:411-423.

27. Beshir KB, Sutherland CJ, Sawa P, Drakeley CJ, Okell L, Mweresa CK, Omar SA, Shekalaghe SA, Kaur H, Ndaro A, et al: Residual *Plasmodium falciparum* parasitemia in Kenyan children after artemisinin-combination therapy is associated with increased transmission to mosquitoes and parasite recurrence. *J Infect Dis* 2013, 208:2017-2024.
28. Four Artemisinin-Based Combinations Study G: A head-to-head comparison of four artemisinin-based combinations for treating uncomplicated malaria in African children: a randomized trial. *PLoS Med* 2011, 8:e1001119.
29. Bousema JT, Schneider P, Gouagna LC, Drakeley CJ, Tostmann A, Houben R, Githure JI, Ord R, Sutherland CJ, Omar SA, Sauerwein RW: Moderate effect of artemisinin-based combination therapy on transmission of *Plasmodium falciparum*. *J Infect Dis* 2006, 193:1151-1159.
30. Witkowski B, Amaratunga C, Khim N, Sreng S, Chim P, Kim S, Lim P, Mao S, Sopha C, Sam B, et al: Novel phenotypic assays for the detection of artemisinin-resistant *Plasmodium falciparum* malaria in Cambodia: in-vitro and ex-vivo drug-response studies. *Lancet Infect Dis* 2013, 13:1043-1049.
31. Chotivanich K, Tripura R, Das D, Yi P, Day NP, Pukrittayakamee S, Chuor CM, Socheat D, Dondorp AM, White NJ: Laboratory detection of artemisinin-resistant *Plasmodium falciparum*. *Antimicrob Agents Chemother* 2014, 58:3157-3161.
32. WHO: Emergency response to artemisinin resistance in the Greater Mekong subregion. *Regional Framework for Action 2013 - 2015*. WHO, Geneva

Chapter 8

Severe malaria not responding to artemisinin derivatives in man returning from Angola to Vietnam

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Abstract

Resistance to artemisinin derivatives, the most potent antimalarial drugs currently used, has emerged in South-East Asia and threatens to spread to Africa. We report a case of malaria in a man who returned to Vietnam after 3 years in Angola that did not respond to intravenous artesunate and clindamycine or an oral artemisinin-based combination.

Artemisinin derivatives are used in combination with other drugs for the treatment of *Plasmodium falciparum* malaria. Nevertheless, *P. falciparum* resistance to artemisinins has been recently detected in four countries (Cambodia, Thailand, Myanmar, and Vietnam) of the Greater Mekong sub-Region [1]. Artemisinin resistance could spread from other countries to other regions, including sub-Saharan Africa where the incidence of malaria is the highest, and where artemisinin resistance would have devastating consequences. Increased and uncontrolled travel between Asia and Africa might contribute to the spread of artemisinin – resistance malaria parasite.

An estimated 40,000 Vietnamese workers travel annually to Angola [2] where *P. falciparum* malaria is the leading cause of illness and death [3, 4]. Over the past year, an increasing number of severe malaria cases have been identified among Vietnamese migrants returning from Angola [5]. During March-mid- April 2013, five deaths from malaria among Vietnamese workers in Angola have been reported [6-8]. We report a case of Vietnamese man who returned from Angola and did not respond to intravenous artesunate or oral artemisinin based combination therapy.

The study

In April 2013, a 58 year-old Vietnamese man was admitted to Bach Mai Hospital in Hanoi, Vietnam because of high fever, jaundice and lack of consciousness. Eleven days before hospitalization, he had returned from Saurimo city in Angola, where he had been working in the construction industry for the past three years, to his home village in the malaria free province of Nam Dinh province, 80 kilometers from Hanoi. Four

days after returning from Angola, he reported fatigue, fever with chills, and a cough. Two days later, he went to the local district hospital where he was given a diagnosis of bronchitis and received a third-generation cephalosporin antibiotic (cefixime) and antipyretics for 3 days. Ten days after his arrival in Vietnam, the patient came to the emergency department of Bach Mai hospital because of continuous high fever (body temperature 39-40°C), dyspnea, and urinary incontinence. He was given fosfomycin for pyelonephritis.

Because his clinical condition worsened rapidly, the patient was transferred the next day to the Infectious Diseases Department in the same hospital because of cerebral malaria was diagnosed. At admission to the department, the patient (weight 58kg) was in a confused state and had a Glasgow coma score of 13/15, generalized convulsions, jaundice, and tachypnea (respiration rate 25 breaths/min), a body temperature of 38.5°C, a blood pressure of 130/80mmHg and pulse rate of 121 beats/min. Clinical examination detected hepatomegaly and cracklings in both lungs. Blood tests showed following results: leukocyte count 13.3×10^9 cells/l; hemoglobin 153 g/l; platelet count 20.9×10^9 /l; blood urea nitrogen 19.8 mmol/l; serum creatinin 135 mmol/l; aspartate aminotransferase 125 IU/l; alanine aminotransferase 45 IU/l, C-reactive protein 16 mg/l; procalcitonin >120 ng/ml; total bilirubin 116µmol/l; direct bilirubin 11.5µmol/l; and a standard levels of electrolytes.

Parasite density/microliter of blood was calculated after counting the total number of *P.falciparum* trophozoites/200 leukocytes and assuming a leucocyte concentration

of 8000 cells/ μl . Microscopy identified *P. falciparum* trophozoites at a concentration of 378,470/ μl and some schizonts. A chest radiograph showed bilateral pneumonia. Analysis of cerebrospinal fluid and a computed tomographic scan of the brain showed standard results. Test results for HIV and hepatitis B surface antigen were negative.

The patient was given a diagnosis of *P. falciparum* cerebral malaria and treated with intravenous (IV) artesunate (batch N°511004; Pharbaco, Hanoi, Vietnam) (60mg every 12h, loading dose 120mg at admission and IV clindamycin (600mg every 12h). After 24h of hospitalization, the patient had a Glasgow coma score of 11, a body temperature of 39.7°C and a parasite density of 329,411/ μl (Figure 1), a blood pressure of 70/40 mmHg, and a respiration rate of 30 breaths/min. An ultrasound examination showed hepatomegaly and bilateral pleural effusion. At day 2 of hospitalization, the patient was treated with intubation and mechanical ventilation. At day 4, the hemoglobin level had decreased to 9.3g/dl and the patient was given a blood transfusion. After 5 consecutive days of treatment with IV artesunate and clindamycin, the patient remained comatose and had continuous fever (39°C) and high parasite density (148,000 parasites/ μl).

At day 6 of hospitalization, artesunate and clindamycin were discontinued and oral treatment with dihydroartemisinin (40mg/tablet)/ Piperaquine (320mg/tablet) was administered through a nasogastric tube for 3 days (4 tablets/day the first day then 2 tablets/day). After 3 days of treatment, the patient was still febrile (38.5°C) and had a Glasgow coma score of 12 and a parasite density of 113,409 parasites/ μl (Figure 1).

Quinine (1,750mg/day) and doxycyclin (200mg/day) were then administered at day 9 through a nasogastric tube (injectable quinine was not available). Twelve hours after the first dose of quinine, parasite density was 55,558 parasites/ μ l. It decreased to 9,668/ μ l 72h at day 11 after starting quinine treatment when fever eventually subsided. Two days later, the patient regained consciousness, and at day 15 of hospitalization, blood slides were negative for parasites (Figure 1). The patient eventually recovered and discharged 35 days after admission.

Species-specific PCR [9] of a blood sample obtained on day 3 of hospitalization confirmed the diagnosis of *P.falciparum* mono-infection. Further genotyping by using nested PCR (merozoite surface protein 1 and 2 repeat markers) and capillary electrophoresis [10] of several filter paper blood spots obtained during the 23 days of hospitalisation confirmed that the patient had a polyclonal *P. falciparum* infection with ≥ 2 clone. These clones might have persisted from admission through day 10 of hospitalization (Figure 2).

The quality of IV artesunate used was determined by using high-performance liquid chromatography according to USP34 NF29 specifications at National Institute of Drug Quality Control (Hanoi, Vietnam) (<http://nidqc.org.vn/>). The drug was found to be acceptable.

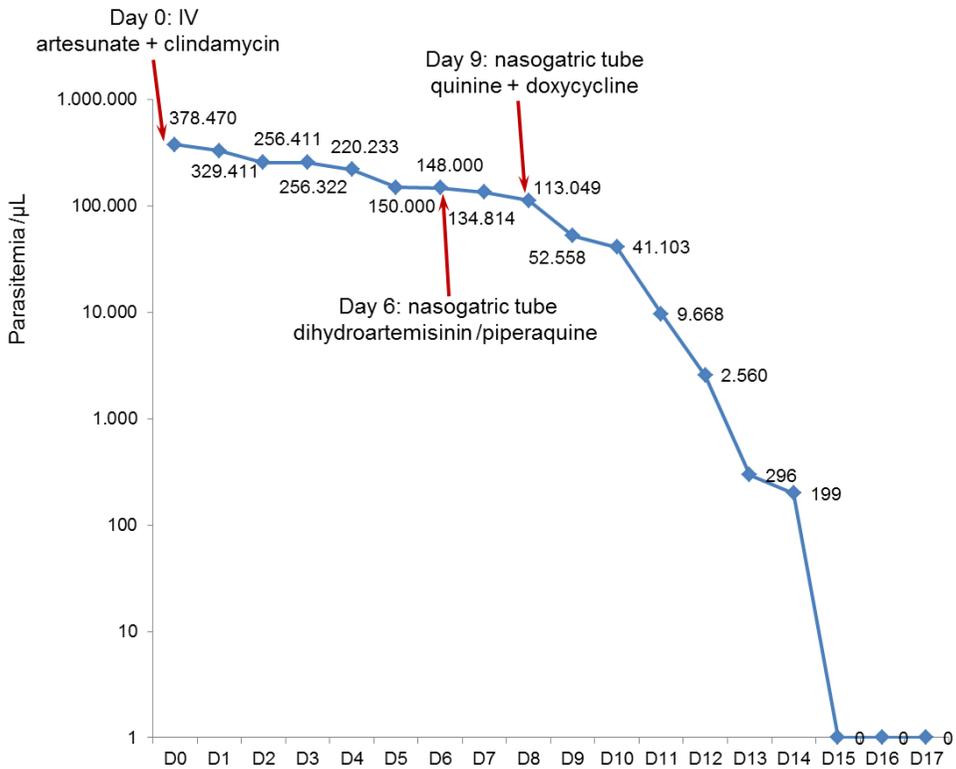


Figure 1. Evolution of *Plasmodium falciparum* parasite density (log scale) by day after start of antimalarial treatments for man with severe malaria who returned from Angola to Vietnam in April 2013.

Values are parasites/microliter of blood. IV, intravenous.

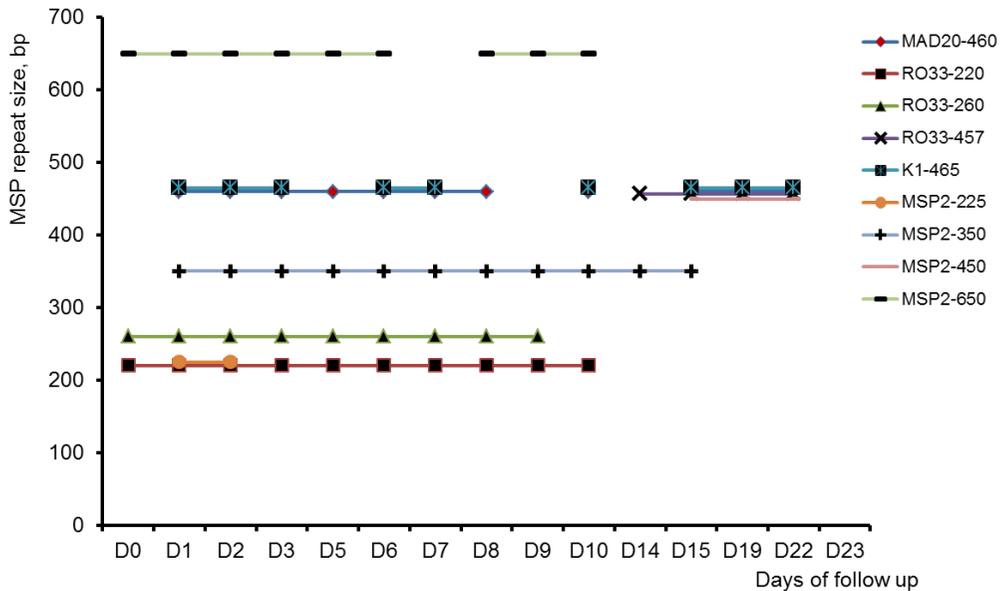


Figure 2. Clonal complexity of *Plasmodium falciparum* strain that cause severe malaria in man who returned from Angola to Vietnam after treatment, as determined by merozoite surface protein (MSP1) and MSP2 repeat length molecular typing. Allele sizes were detected by capillary electrophoresis of amplified MSP repeat regions and shown for each follow up sample.

Conclusion

This clinical case of suspected artemisinin resistance originating from sub-Saharan Africa is of concern because the patient had probably been infected in Angola and because clinical presentation which differs delayed parasite clearance reported in Southeast Asia [1]. The patient never had malaria and had worked continuously in Angola for the past 3 years before his return (direct flight to Hanoi then directly by car to his home village) to a malaria free area in Vietnam. Given the onset of symptoms only 4 days after his return, the patient was most likely infected in Luanda Sul province,

Angola, to which malaria is hyperendemic and where Vietnamese workers have reportedly died of malaria [6-8].

Five days of treatment with intravenous artesunate and clindamycin and a 3-day regimen of dihydroartemisinin/piperaquine did not clear the infection because parasite density remained high (>100,000 parasites/ μ l) until the eighth day of hospitalization. Parasite density showed a logarithmic decrease only after treatment was changed to quinine and doxycycline.

An external quality control of all blood slides was performed by an expert microscopist at the National Institute of Malariology, Parasitology and Entomology, Hanoi; the parasite densities were confirmed. Therefore, this case is suggestive of *P. falciparum* tolerance to artemisinin derivatives; however because no blood samples for pharmacokinetic or *in vitro* studies were collected, resistance could not be confirmed.

Identification of *P. falciparum* malaria parasites tolerant to arteminins raises serious concerns that artemisinin-resistant strains have emerged or spread in Africa. This finding would be a major public health disaster and needs to be urgently confirmed by larger treatment efficacy studies in Angola.

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References

1. World Health Organization. Joint assessment of the response to artemisinin resistance in the greater Mekong sub-region. Conducted November 2011 to February 2012. Summary report. (<http://whothailand.healthrepository.org/bitstream/123456789/1484/3/120418-Artemisinin-Resistance-in-GMS-SummaryReport.pdf>). Last accessed on 11 Dec.2013.
2. Dan Tri (official Journal of the Vietnamese government). Vietnamese workers are tempted by high income in Angola. Intellectuals' magazine. Interview of Prime Minister Mr Le Thanh Hoa – A Deputy Minister of Labour, Invalid and Social Vietnam. (<http://dantri.com.vn/xa-hoi/lao-dong-viet-bi-cam-do-boi-muc-thu-nhap-cao-o-angola-733732.htm>). Last accessed on 22 Sep.2013.
3. Measure DHS. Angola malaria indicators, survey 2011 (<http://www.measuredhs.com/what-we-do/survey/survey-display-395.cfm>) Last accessed on 26 Nov.2013.
4. USAID/PMI. Malaria situation in Angola (Dr. Fancisco Saute). http://www.google.be/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&ved=0CCcQFjAA&url=http%3A%2F%2Fangolafieldgroup.files.wordpress.com%2F2008%2F03%2Fmalaria_angola2.pdf&ei=csrBUvX4Aqed0AXR14DQBA&usg=AFQjC NFocMi 3L7wJJzRXERv3EeQdlq6iw&bvm=bv.58187178,d.d2k. Last accessed on 11 Dec 2013.
5. Health and Life - Vietnam Ministry of Health website. Imported malaria warning. <http://suckhoedoisong.vn/y-te/canh-bao-sot-ret-ngoai-nhap-khang-thuoc-20130814091119352.htm>. Last accessed on 1 Dec.2013.
6. Institute of Malariology, Parasitology and Entomology, QuyNhon, Vietnam. What can be seen from Vietnam workers who died of malaria in Angola? (<http://www.impe-qn.org.vn/impe->

- [gn/vn/portal/InfoDetail.jsp?area=58&cat=944&ID=6382](http://vn/portal/InfoDetail.jsp?area=58&cat=944&ID=6382)). Last accessed on 18 Apr.2013.
7. Dan Tri (official journal of the Vietnamese government). A Vietnamese worker died of complicated malaria in Angola. (<http://dantri.com.vn/xa-hoi/them-mot-lao-dong-viet-tu-vong-vi-sot-ret-ac-tinh-o-angola-742723.htm>). Last accessed on 14 Aug.2013.
 8. One more Vietnamese male worker died of malaria in Angola. Education magazine (<http://giaoduc.net.vn/Xa-hoi/Phat-hien-them-mot-nam-lao-dong-chet-vi-sot-ret-tai-Angola/299241.gd>).Last accessed on 30 Nov.2013.
 9. Rubio JM, Post RJ, van Leeuwen WM, Henry MC, Lindergard G, Hommel M: Alternative polymerase chain reaction method to identify Plasmodium species in human blood samples: the semi-nested multiplex malaria PCR (SnM-PCR). *Trans R Soc Trop Med Hyg* 2002, *96* (1 Suppl): S199—204.
 10. LijandeA, Wiklund L, Falk N, Kweku M, Martensson A, Felger I, Farnert A: Optimization and validation of multi-coloured capillary electrophoresis for genotyping of *Plasmodium falciparum* merozoite surface proteins (msp1 and 2). *Malar J* 2009, *8*:78.

In response

**Severe malaria not responding to artemisinin derivatives
in man returning from Angola to Vietnam**

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We agree with Ringwald and Dondorp [1] that our report of a Vietnamese worker returning from Angola with severe *Plasmodium falciparum* malaria not responsive to artemisinins [2] is unlikely to indicate that artemisinin resistance has reached Angola. Nevertheless, this case, for its unusual clinical manifestation and response to treatment, had raised alarm in Vietnam, where the number of imported malaria cases and deaths among Vietnamese workers returning from Africa has recently increased [3, 4]. After our report was published in July 2014, we collected additional information that may be useful in putting such a case in perspective.

The results of an external quality control study by Sigma-Tau Pharmaceuticals on 10 vials of the same batch (no. 511002) of intravenous artesunate as administered to our case-patient (report available on request) confirmed acceptable drug concentration and showed that the opalescence observed after reconstitution was caused by precipitation of an impurity (representing 0.12% of the preparation) identified as an active metabolite of artesunate. Therefore, the treatment administered to the patient was of acceptable quality. The blood concentrations of artesunate and dihydroartemisinin may have been 20% lower than ideal (as predicted by a pharmacokinetic model), but this finding cannot explain why the parasite density remained $>200,000/\mu\text{l}$ for several days.

Ringwald and Dondorp also mention functional asplenia as a possible cause of delayed parasite clearance. We argue that this would have resulted in a much longer (weeks/months) parasite clearance [5] than the observed sharp decrease after quinine

and tetracycline administration. Moreover, we did not observe any accumulation of circulating dead parasites (Howell-Jolly bodies), which is against the hypothesis of functional asplenia. Furthermore, sharp decline of parasite density immediately after quinine and doxycycline administration by nasogastric tube is not consistent with the proposed hypothesis of reduced intestinal absorption.

In hindsight and after consideration of additional information, we agree that it is unlikely this patient harbored several resistant parasite clones. However, the reasons for the lack of response to arteminins in this patient remain unknown and are under continued investigation.

The discussion triggered by the publication of our case report raises the question of what should be reported to the attention of the scientific community and public health authorities. Besides being an obligation for clinical physicians, reporting unusual treatment failures such as our case is also an essential component of antimalarial resistance surveillance. As mentioned by Ringwald and Dondorp, “vigilant monitoring is pivotal” for the detection of possible foci of resistance. For early detection of artemisinin resistance, we would rather have a more sensitive than specific system, because the latter would probably miss the first emerging cases of resistance.

Reporting cases similar to the one we published should be encouraged.

References

1. Ringwald P, Dondorp AM: Several malaria not responsive to artemisinin derivatives in man returning from Angola to Vietnam. *Emerg Infect Dis* 2015, *21*:1264-65.
2. Van Hong N, Amambua-Ngwa A, Tuan NQ, Cuong do D, Giang NT, Van Dung N, Tinh TT, Van Tien N, Phuc BQ, Duong TT, Rosanas-Urgell A, D'Alessandro U, Van Geertruyden JP, Erhart A: Severe malaria not responsive to artemisinin derivatives in man returning from Angola to Vietnam. *Emerg Infect Dis* 2014, *20*(7):1199-202.
3. National Institute of Malariology, Parasitology and Entomology (NIMPE): Annual report of the National Malaria Control Program in Vietnam of 11 months in 2013. Hanoi (Vietnam). Internal report 2013.
4. National Institute of Malariology, Parasitology and Entomology (NIMPE): Annual report of the National Malaria Control Program in Vietnam: 2014. Hanoi (Vietnam): Internal report 2015.
5. Chotivanich K, Udomsangpetch R, McGready R, Proux S, Newton P, Pukrittayakamee S, Looareesuwan S, White NJ. Central role of the spleen in malaria parasite clearance. *J Infect Dis* 2002, *185*(10):1538-41.

Chapter 9

General discussion

In this thesis, we have combined different types of epidemiological studies. We used molecular tools, in order to quantify the human reservoir of malaria accurately and to investigate genetic diversity and population structure of *P.vivax* parasite populations in central Vietnam. We confirmed the spread of *P. falciparum* parasites resistant to ACT in the region and generated relevant knowledge not only to better understand the current challenges of malaria elimination efforts in the region, but also to inform health authorities and contribute to the implementation and use of molecular tools in elimination campaigns, epidemiological research and surveillance efforts in the country.

Translation of research outcomes into health policies

Several outcomes of this thesis have contributed to changes in the country's health policies. First, *P.knowlesi* treatment was included in the national treatment guidelines [1] after the description of the first detection of *P.knowlesi* infections in humans in Ninh Thuan province [Chapter 4]. In addition, our report describing an important rate of *P.malariae* and *P.ovale* infections and mixed infections usually non-detectable by LM prompted the NMCP to reinforce LM training with the aim of improving skills to identify non-*falciparum* *Plasmodium* species (*P.vivax*, *P.malariae*, *P.ovale* and *P.knowlesi*) and mixed infections (Chapters 4 and 5) [2, 3]. Since 2014, the NMCP has also incorporated ss-PCR in its surveillance programme, in order to accurately monitor

malaria transmission in Vietnam [4-7]. In 2016, the Molecular Biology Department at the NIMPE received ISO15189 accreditation for the ss-PCR technique [8, 9].

Furthermore, our report on *P.falciparum* resistance to artemisinins in Quang Nam in 2013, prompted the NMCP and WHO to declare this province as a “Tier I area”, *i.e.* an area with “credible evidence of artemisinin resistance” [10]. Consequently, the financial support received by the Quang Nam provincial malaria center (from different projects) increased from 150,000 USD in 2013 to 786,379 USD in 2014. This additional funding supported the implementation of intensified control strategies based on vector control activities (ITNs and IRS), PCD at community level, reactive case detection and treatment, as well as regular drug efficacy studies supported by WHO in sentinel sites. These efforts were followed by a strong reduction of malaria morbidity, with a 75% reduction within one year [2, 11, 12].

Finally, the case report of a lack of response to intravenous artesunate and DHA-PPQ treatment of a Vietnamese worker with severe malaria returning from Angola urged the NMCP to introduce WHO recommendations in national guidelines for the treatment of severe malaria and to strength quality control of anti-malaria drugs [1].

High proportion of asymptomatic and sub-microscopic infections

In terms of epidemiology, our study in Ninh Thuan evidenced the high sensitivity of PCR in detecting malaria infections compared to LM, in a region where malaria transmission has greatly decreased in recent years and where a majority of infections are now asymptomatic and harbour low parasite densities [Chapter 5] [2, 7]. Using a

SnM-PCR [Chapter 2], we detected an overall prevalence of malaria by PCR of 29.1% compared to 14.4% by LM, while the majority of PCR-detected-infections were asymptomatic (~80%). In addition, PCR revealed a non-negligible prevalence of *P.malariae* and *P.ovale* infections (4.1% and 0.8%, respectively) that were not previously detected by LM and 7.1% of mixed infections. PCR detected high proportions of asymptomatic infections in Quang Nam province in 2009 (N=327) and Quang Tri province in 2010 (N=1,390) accounting for 75.4% and 98.1% of all infections respectively [13, 14]. In addition, a *P.malariae* prevalence of 1.2% as detected by PCR was observed in Quang Binh province (2004-2005) when analysing 3,627 individuals positive by LM; this study did not detect *P. ovale* infections [15]. On the other hand, several cross-sectional surveys, conducted from 2003 to 2014 in provinces from Central and Southern Vietnam used PCR-based methods to measure species distribution among LM patent infections and fever cases and reported variable PCR-based prevalence of *P.malariae* and *P.ovale* infections; this was respectively, i.e. 0.37% and 0.07% in Binh Phuoc (N=2,635), 0.5 and 0% in Ninh Thuan (N=3,405), 2.03% and 0.41% (N=2,167) in Gia Lai between 2003-2007 [16] or 6.7% for both *P. malariae* and *P. ovale* in Quang Tri (N=1,390) in 2010 [14] and 2.9% and 1.4% in Southern Vietnam in 2014 (N=494) [17]. A high proportion of asymptomatic infections have been detected in several provinces in Vietnam [13, 14] and the prevalence of mixed infections and of the minor *Plasmodium* species (*P. malariae* and *P. ovale*) varied between studies and provinces [13, 14, 16]. Some of these differences can be attributed to different study designs, individual inclusion criteria [14-18] and the use

of different diagnostic tools as mentioned above. Most of the studies conducted at the local level and by the NMCP relied heavily on LM or RDTs missing an important proportion of asymptomatic, mixed and *P.malariae* and *P.ovale* infections [2, 3, 7, 19, 20]. While our study in Ninh Thuan screened a large population (n=2,303) from several communities (10 communes and 30 villages), thus warranting a high representativeness and precision of our prevalence estimates. A high proportion of asymptomatic infections and thus a large parasite reservoir were also observed in other GMS countries i.e. Cambodia, Thailand and Myanmar, where malaria transmission is low, and NMCPs are also moving into malaria elimination strategies [21-23].

Malaria cases in Vietnam are now concentrated in 10 provinces from the centre of the country and the highlands, which is associated with behavioral risk factors in particular populations [2, 7]. On the other hand, even in provinces where malaria transmission has been greatly reduced [13, 14], there is a non-negligible population of malaria vectors (i.e. *An. dirus*, *An. minimus*, *An. maculatus* etc.) [24], which poses a risk of resurgence in these areas.

In addition, detection of submicroscopic infections is a challenge to current elimination efforts, as the most common used diagnostics tools are LM and RDT. Furthermore, quality of LM results varies within different levels of the health system with most experienced microscopists located in central laboratories in major cities, while the majority of malaria cases concentrate in rural areas. Lack of proper

infrastructure in peripheral health posts confront patients with unacceptable delays in diagnosis [25, 26]. On the other hand, RDTs are useful tool for case management in rural areas where LM is not available. Since 2005, the NMCP has used different type of RDTs, e.g. Paracheck F test (Orchid Biomedical Systems, Goa, India), ParaSight F test (Becton Dickinson Diagnostic Systems, Cockeysville, Md.), SD Bioline, malaria Ag Pf/Pv (Standard diagnostic, INC, Korea) and ICT P.f/P.v (ICT-Amrad, Sydney, Australia) [27, 28].

Even though high sensitivity RDTs for the detection of *P. falciparum* infections have been developed and are now in the testing phase [29, 30], RDTs used in Vietnam are are less sensitive than LM and thus, miss most infections with parasite densities under 100 parasites/ μ l [27], while unable to detect *P.malariae* and *P.ovale* infections [27, 28]. Consequently, malaria infections are underreported at the NMCP level. This confirmed in several studies showing a high proportion of sub-patient infections in several provinces in Vietnam such as Ninh Thuan (50.7%) [Chapter 5], Quang Nam (58.7%) [13] and Quang Tri (98.1%) [14]. Pf-HRP2 deficient parasites leading to false negative RDT results, have not been reported in Vietnam [31], however, an exhaustive analysis with a representative samples size and geographical areas or regular monitoring is urgently needed after deficient parasites have been reported in South East Asia [32].

A systematic review of studies carried out between 1998 to 2012 in 39 sites across 22 countries showed that the proportion of *P.falciparum* and *P.vivax* asymptomatic

infections detected by PCR ranged from 0% to 16.8% in low-, 9.6% to 58.9% in moderate-, and 16.3% to 82.5% in high transmission areas, with all age groups affected [33]. A review of 44 cross-sectional surveys showed that an average of 55.7% of *P. falciparum* and 69.5% of *P. vivax* infections are only able to be detected by PCR in low transmission settings [34]. Another large cross-sectional survey in the Thailand–Myanmar border areas, Cambodia, and Vietnam also showed that only 4% and 5% of malaria infections were detected by RDT and LM, respectively, while the number reached 20% when using high volume ultra-sensitive real-time polymerase chain reaction [35]. A review of 106 different surveys estimated that asymptomatic and submicroscopic infections account for 20–50% of all human-to-mosquito transmissions [36]. In our study [Chapter 5], the prevalence of LM-positive samples harboring gametocytes was estimated at 6.3% (144/2,303), which is underestimated due to the limited sensitivity of LM-based gametocyte detection compared to molecular tools. Indeed, studies using RT-qPCR tools can detect *P. falciparum* gametocyte densities as low as 0.3 gametocytes/ μl [37], estimating gametocyte prevalence 10-20 times higher than those detected by LM [38, 39]. Previous studies have demonstrated that mosquitos can be infected with *P.falciparum* gametocyte densities as low as 5 gametocytes/ μl or even undetectable gametocyte densities (by QT-NASBA), which in naturally infected Kenyan children contributed to infect up to 10% of all infected mosquitos [40]. In a recent study from Colombia on the infectivity of *P.vivax* to mosquitos, the majority of *P.vivax* infections (including asymptomatic and submicroscopic) harboured gametocytes, and the highest proportions of mature

gametocytes were observed in asymptomatic patients (presumably long-lasting infections), who were as infective as acutely ill patients [41].

Thus, to better understand malaria transmission in a given area it is important to accurately characterize the human parasite reservoir (including submicroscopic infections and species distribution) and determine the proportion of infections carrying gametocytes, as well as to identify the risk factors for gametocyte carriage in the population. To this aim, molecular diagnosis tools are much more sensitive methods to detect malaria infections, identify species and sexual stages than still routinely used RDT and LM, which miss a high proportion of infections and lead to an unreported malaria burden [36].

In summary, the modified SnM-PCR [Chapter 2] demonstrated to be a suitable method for the identification of the five human *Plasmodium* species in Vietnam, with high sensitivity and an acceptable cost. It is, however, still difficult to perform as a Point of Care test (POC) due to the lack of laboratory equipment and scientific staff [13, 14]. Even at the central level, funds are still limited to run molecular tests systematically on large population surveys in sentinel sites (only filter paper blood samples collected from suspected and confirmed malaria patients in 7/64 provinces are analysed by ss-PCR [4-7]). Current strategies of the NMCP in Vietnam have changed to active case detection, even though the diagnostic tool of use continues to be RDT/LM; our results show that inclusion of molecular tools in the NMCP will increase the impact of the intervention.

In this regard, the development of a LAMP diagnostic tool that can be performed at the point of care (POC), and with a sensitivity equal to or higher than PCR-based methods, may become a promising tool for malaria elimination campaigns [42]. Different versions of the LAMP assay (including recent ones with a high throughput format) have been evaluated in malaria pre-elimination regions such as Zanzibar [43, 44], and are currently under evaluation in Vietnam (NIMPE-FIND collaboration). If field evaluation is successful, LAMP could become an interesting alternative to PCR for the active detection of malaria infections in remote-rural areas as part of malaria elimination campaigns in Vietnam.

Finally, though not mentioned in the different chapters of this thesis, NIMPE is currently implementing RT-qPCR methods for the detection and quantification of *P. falciparum* and *P. vivax* gametocytes, both in clinical trials and epidemiological studies.

***P. knowlesi* in Vietnam**

Our first identification in 2009 [Chapter 4] of *P. knowlesi* infections in three young asymptomatic patients in a Rag lai (minority) community of Ninh Thuan province, together with three other reports of *P. knowlesi* infections both in *An. dirus* and in asymptomatic humans (also from the Raglai minority group) in the neighboring Khanh Hoa province [45-47], raises the issue of simian malaria transmission among ethnic minorities living in forested areas of Central Vietnam, and its threat to current elimination efforts. Indeed, even if human malaria could be eliminated, the zoonotic transmission of *P. knowlesi* will be maintained by the presence of the main vector *An.*

dirus and the macaque monkeys. Given the permanent close contact between some indigenous populations and the forest, the re-introduction of *P. knowlesi* into the human population will remain a permanent threat. Though *P. knowlesi* infections have not yet been identified in Vietnamese monkeys, *P. inui*, another simian species infecting humans, was identified in one pig-tailed macaque and one long-tailed macaque in Binh Phuoc province [48], where *A. dirus* is the predominant vector species [24].

Further large-scale surveys are urgently needed in forested areas of Central Vietnam to assess the geographical extent, clinical and parasitological characteristics of *P. knowlesi* infections in the human host. To this aim, (molecular) diagnostic tools able to accurately identify *P. knowlesi* infections and that could be implemented in surveillance systems are urgently needed due to the limited sensitivity and specificity of LM and RDTs [49].

As part of this PhD [Chapter 3], we extended a previously designed SnM-PCR for the detection of 4 human malaria species [50] to specifically detect *P.knowlesi*. Therefore, a two stage SnM-PCR for the detection of the five human malaria species has been validated and is currently implemented at the NIMPE as part of the malaria surveillance system in sentinel sites or within research projects. We used this protocol to investigate the prevalence of *P.knowlesi* in previous malariometric surveys carried out in Gia Lai (2008) and Quang Nam (2009) provinces, but no new cases were detected (N.V. Hong, *unpublished data*). Alternative tools include surface protein

containing an altered thrombospondin repeat domain (SPATR), considered as a biomarker for immunoblot and ELISA-based assays [51] and the LAMP assay for detecting *P. knowlesi* malaria parasites in the field is also available [52]. *P. knowlesi* infections in humans, have also been reported in Thailand and Myanmar, which have similar forested environments [53, 54], in the Philippines [55], Singapore [56], Indonesian Borneo [57], Malaysia [58] and Cambodia [59], raising concerns regarding the spread of this pathogen in humans in Southeast Asia. Therefore, control and elimination programmes in the Greater Mekong Sub-Region should take into account *P. knowlesi* infections and its zoonotic reservoir in their elimination goals.

Population genetics

The high prevalence of sub-microscopic infections identified in several endemic areas of Central Vietnam [Chapter 5] [13, 14] reflects the decrease in malaria transmission as a result of the continued and comprehensive control interventions applied since 1992 following the WHO recommendations.

There are some key biological differences between *P. falciparum* and *P. vivax* parasites that makes the latter species more difficult to control with conventional methods [60]. These include the occurrence of relapsing liver forms, the early gametocyte production before the onset of symptoms [61] as well as the usually low parasitemias, all of which facilitate the undetected transmission of *P. vivax* and challenge its effective treatment and control [60]. It is now clear that malaria elimination efforts cannot progress further without an in-depth knowledge of the epidemiology of both

parasite species and respective host-parasite-vector interactions. In addition, tools to accurately measure the differential impact of intensified interventions on both *P.vivax* and *P.falciparum* transmission are needed [62].

Molecular epidemiology studies aiming at understanding genetic diversity, transmission dynamics and population structure of parasite populations both at local and global levels, can greatly contribute to our understanding of the evolution of parasite populations, their interactions with human hosts, to measure changes in transmission, and guide the development and implementation of new interventions [63-67].

Our study [Chapter 6] describes in detail the genetic diversity, population structure of *P. vivax* infections identified in 4 rural communities of Central Vietnam before radical treatment and inclusion in a 2-year follow-up study. We found moderate levels of heterozygosity ($He=0.68$) and little population structure. These results contrast with a previous study conducted in neighboring Binh Thuan province, where levels of genetic diversity were higher (He ranged from 0.84 to 0.86) [68] despite similarly low levels of malaria transmission [66, 68]. On the other hand, similar levels of genetic diversity and little population structure have also been described in other GMS countries now aiming to eliminate malaria, such as Cambodia ($He=0.87$), Lao PDR ($He=0.83$), Thailand ($He=0.89$) as well as in India ($He=0.9$) [66, 69].

In areas of low transmission, *P.falciparum* populations often display a low proportion of polyclonal infections and a high linkage disequilibrium (LD) [70, 71]. However,

P.vivax parasite populations do not follow this correlation, and high proportions of polyclonal infection are also found in areas of low transmission and in parasite populations with significant LD [68, 72, 73]. In Vietnam, *P.vivax* population structure studies are scarce but high levels of inbreeding and significant LD were reported from both Quang Nam and Binh Thuan provinces [Chapter 6] [68]. High *P.vivax* genetic diversity found in countries with relatively low transmission highlights the complexity of the parasite populations and consequent challenges to elimination [66, 68, 69, 74].

In 2014, the genetic diversity of *P.vivax* isolates collected in four different continents was analyzed using a common panel of MS that were chosen after testing by Lositan software. The highest genetic diversity was found in Southeast Asia, while intermediate diversity was found in the South Pacific and the lowest in South America and Central Asia [66]. This study highlights that in areas such as Southeast Asia and South America with similar malaria transmission intensity, the level of genetic diversity varies substantially, indicating that besides climatic and topographical factors, similar intervention policies and strategies *e.g.* impregnated bed-nets, insecticide spraying, ACT treatment etc. [75] may affect parasite populations of distinct geographical regions differently [69].

Our study [Chapter 6] showed that the *P.vivax* population in this remote forested area of Central Vietnam presented a high level of relatedness and a high rate of polyclonal infections (71.3%). High polyclonality increases the probability of genetic recombination during the sexual cycle in the mosquitoes, resulting in sporozoites with

novel but related genotypes [76]. As in *vivax* populations, some variants in the complex mixture are more prone to relapse than others [77]; the high genetic relatedness may help us to recognize genotypic patterns of relapsing parasite populations and identify individuals at greater risk for relapsing malaria [77, 78]. A preliminary analysis of the monthly follow up samples of our *P.vivax* cohort have shown a high rate of recurrent vivax infections, which will be further analyzed in terms of genetic relatedness.

P.vivax hypnozoites can produce multiple relapse during which individuals even with low parasite densities may still remain infectious to mosquitos [79]. Currently available diagnostic tools cannot detect hypnozoite stages. Therefore, *P.vivax* relapses are important contributors to the maintenance of malaria transmission [80, 81]. This was also suggested in our study in central Vietnam, where a high MOI was found, contrasting with what would be expected in an area of low transmission, and this was also the case in previous reports from low transmission areas such as central Thailand (MOI=1.69) [61, 82], northwest Thailand (MOI=3.0), southern Cambodia (MOI=2.9) [83] or Myanmar (MOI=1.87) [61]. Asymptomatic infections and relapses may contribute to this high rate of polyclonal infections, as has been described in different regions of the world [84, 85].

In a high transmission area such as Papua New Guinea, it was estimated that four of every five *P.vivax* infections in children from 5 to 10 years was due to relapses [80]. The relapse rate of *P.vivax* varies across geographic regions and is partially dependent

on immunity of the subjects [79, 86]. Currently, the only available treatment for *P. vivax* radical cure is a full 14-day PQ course [87]. Unfortunately, this treatment poses great challenges for *P. vivax* elimination efforts in countries like Vietnam, due to low adherence to the 14-day regimen and the risk of hemolysis in G6PD deficient patients while G6PD POC diagnosis is not available in the country [88].

In our study, we genotyped *P. vivax* MS by PCR amplification followed by capillary electrophoresis (CE). This method has the advantage compared to agarose gel analysis of a higher resolution, since differences of 1-2 bp can be identified. On the other hand, disadvantages of this method are the difficulty to detect minority clones in a single infection or non-perfect identification of clones due to low parasite density [66]. It was shown in our analysis on recurrent infections that MS positivity was reduced due to a high rate of asymptomatic and submicroscopic infections (unpublished data). This phenomenon can be partially improved by analyzing a second sample from the same patient 24h after the first one, which has been shown to increase the number of clones that can be detected [89]. The study showed that only markers with balanced diversity should be used for decoding population diversity, and structure as well as population comparisons in different regions and the markers that have extreme excess diversity, can provide meaningful properties for MOI studies [90]. In order to differentiate between relapse and new infections in drug efficacy trials, highly polymorphic markers with many alleles at moderate frequencies are required [91]. Our study, together with the study in Binh Thuan province, can be considered as pilot studies to assess

individual marker profiles before conducting large-scale genotyping studies in malaria endemic areas in Vietnam. Instead of 14 MS, later studies can reduce the panel of markers (from 2 to 9 MS, depending on the purpose of the studies) [66, 90] but still have the ability to decipher population structure in different areas as well as parasite migration and evolution in Vietnam.

Although new technologies for genotyping and genome analysis are developing quickly, they still require sophisticated laboratory facilities and bioinformatics skills that are not yet available in most research institutes in limited resource settings, such as the NIMPE in Vietnam. Therefore, MS genotyping remains a useful tool for investigating genetic diversity and population structure of *Plasmodium* populations in Vietnam. However, the choice of MS is dependent on the research question and should be done in accordance with local genetic diversity data, and if possible MS should be tested in a pilot study with local samples. In the near future, population genetic studies and surveillance on drug resistant markers should be implemented as molecular surveillance tools to continuously evaluate the impact of elimination efforts in the country and inform and support current strategies and challenges [92, 93]. For the NMCP in Vietnam, we recommend the use of MS markers to re-evaluate *P. vivax* in Binh Thuan or Quang Nam, in order to establish how genetic diversity and population structure changes in the condition of decreasing transmission.

Drug resistance

Vietnam's current malaria elimination efforts, as in most GMS countries, are being threatened by the development of *P. falciparum* resistance to artemisinin derivatives [94, 95]. Alarming rates of D3 positivity (15.6%) were reported for the first time in Binh Phuoc province in 2009 [96, 97], and by 2016 up to six provinces in Central Vietnam were classified as a delayed parasite clearance area [98]. In 2012, our study in Quang Nam province [Chapter 7] identified a high D3-positivity rate (29.2%), an extended parasite clearance slope (6.2h), as well as a significant association between delayed parasite clearance and the presence at day0 of the SNP 543 of the K13 propeller domain. Interestingly, the I543T mutation was found in the majority of infections (80.7%) while the C580Y was absent, a somewhat different picture than what was found in Binh Phuoc province in 2014 or 2015 where the C580Y mutation was the predominant allele (47.69% [98, 99] and 72.73%, respectively [95]). In addition, another study conducted in Binh Phuoc and Dak Nong provinces from 2010 to 2014, reported 13.89% of C580Y, 52.87% of P553L and 33.33% of V568G mutations [100] or 79.1% and 63% of C580Y in Binh Phuoc and Gia Lai province in the study conducted in 2009-2016 [101]. In Ninh Thuan province, the area has only recently (2015) confirmed a delayed parasite clearance of >10% [98], the K13 mutation accounting for 6% (including I543T, T493H and C580Y) [101]. The difference in K13 mutation rate in Binh Phuoc may have been due to this province having the highest malaria prevalence, as

well at it being the area where artemisinin have been used for the longest period in Vietnam [7, 94]. Moreover, Binh Phuoc and Gia Lai has long borders with Cambodia where artemisinin resistance is endemic, and regular cross-border movements by migrant workers are likely to favor the spread of resistant parasite strains [97, 102] while Quang Nam and Ninh Thuan province, population often travel to forest inside of provinces [13, 101]. In 2016, piperaquine resistance was confirmed in Binh Phuoc [95, 98], and this might be another contributor to the development of artemisinin resistance [97]. In fact, the analysis showed that P553L parasite might have originated independently in Binh Phuoc [101]. In addition, this province attracts annually thousands of seasonal workers from various provinces in Vietnam, and before 2013, self-treatment was encouraged by the NMCP for these migrants into a malaria endemic area [103, 104]. It has been reported by the NMCP that self-treatment with anti-malarial drugs purchased from private pharmacies was more frequent in Binh Phuoc than in other provinces [2, 7]. This may result in repeated inadequate treatment regimens and use of poor quality medicines, practices that favor the development and spread of resistant parasites.

The level of K13 mutation is quite different in the different regions. Among five countries that confirmed artemisinin resistance in SEA, a high prevalent of C580Y mutation was found in Cambodia, Myanmar and eastern and western Thailand, while the F446I mutation is predominant in China-Myanmar border regions as well as in Myanmar [105-108]. Some K13 mutations (examples A578S, K189) have a low

frequency in SEA but presented a high prevalence in Africa [109-111]. However, the most common *k13* mutant alleles (C580Y, I543T, R539T, and Y493H) were found in countries in SEA, indicating that cross-border movement of *P.falciparum* may have already occurred [105]. If study sites were located near international borders, larger population movement across these border areas is possible, which may assist in the spread to a larger extent. In our study in Quang Nam province, the study site is quite isolated with no international border hence parasite resistance might rise independently. Therefore, besides clinical and laboratory investigations to complement sentinel surveillance, the implementation of molecular surveillance to monitor the spread and emergence of resistant parasites is required in Quang Nam.

A recent study in Northwest Cambodia showed that treatment failure was associated with the presence of a triple mutant (including the Kelch 13 C580Y) [102], and the presence of increasing numbers of K13 mutations was associated with high survival rates in novel piperazine *in vitro* assays. In our study [Chapter 7], isolates with delayed parasite clearance had a significantly higher PPQ-IC₅₀ (*ex vivo* maturation assays) than those with normal clearance times, although PPQ-IC₅₀ remained below the cut off of >300 nM for piperazine resistance [112, 113]. Among six provinces that were considered as foci of delayed parasite clearance in Vietnam, a high treatment failure rate (28%) after treatment with DHA-PPQ was only found in Binh Phuoc province, with other provinces having low LTFs (0% in Gia lai, 4% in Ninh Thuan [95], 2.27% in Quang Nam [Chapter 7], 4.3% in Khanh Hoa [114], and 0% in Dak Nong

[115]). This strongly suggests that piperazine resistance has arisen upon a background of artemisinin resistance reported recently from Cambodia [116]. An *in vitro* and molecular study in Binh Phuoc reported the piperazine survival assay rate as being much higher in the parasites from recrudescence infections (39.2%) compared with 0.17% in patients cured with DHA-PPQ and all LTFs related to K13 mutations [95]. It is worth noting that artemisinin-resistant K13 mutants with *ex vivo* PSA survival rates $\geq 10\%$ were associated with a 32-fold higher risk of recrudescence [117]. In Vietnam, confirmatory artemisinin resistance studies that include both K13 prevalences and *ex vivo* data are only available in Binh Phuoc [95, 101] and Quang Nam provinces [Chapter 7], molecular markers of piperazine resistance also only involve screening in this province; therefore, a large-scale surveillance of parasite fitness and *in vitro* drug sensitivity is urgently required.

Although most patients with delayed parasite clearance are cured with ACTs in most of the GMS countries, artemisinin resistance has not been contained despite increasing regional containment efforts. Indeed, resistance to artemisinin continues to spread rapidly across Southeast Asia [118]. The main concern is that artemisinin resistance, with the current increasing population movements, will follow the same path as for other drugs (chloroquine, Sulfadoxine-pyrimethamine etc.) and spread to the African continent, which would be a public health disaster [107, 119].

Although DHA-PPQ remains efficacious in some malaria endemic areas in Vietnam, however effectiveness of drug associated with artemisinin resistance and this places

greater selection pressure on the partner drug. The NMCP has only recently (2016) added a combination of artesunate and mefloquine as the first-line treatment for *P.falciparum* in 5 provinces that have evidence of artemisinin resistance (except Ninh Thuan) [120]. However, due to financial constraints, this policy has not been brought into practice. Therefore, strategies for deployment of multiple first-line therapies are required when effective alternatives are available on the market [121]. Since the currently used first-line treatment (DHA -PPQ) in Vietnam does not meet the WHO criteria of being “based on an average cure rate of >95%, as assessed in clinical trials”, there is an urgent requirement to evaluate alternative treatments with other available forms of ACT to replace DHA-PPQ in the near future.

Case report from Angola

The case report discussed in Chapter 8, despite the fact that artemisinin resistance could not be confirmed, illustrates well the current situation of this permanent threat over the African continent, and how the first emerging cases of artemisinin resistant *P.falciparum* malaria might sooner or later be reported. Indeed, the globalized economic market and facilitated international transport means that in the past twenty years, Asian countries, especially China [122] but also Vietnam [123], are increasingly investing and driving their own labor forces into the African continent. This generates a regular flux of overseas workers returning to their homeland from malaria endemic areas, representing an increasing source of imported malaria cases and deaths [124]. In Vietnam, overseas imported malaria cases especially from Africa have been

increasing since 2013, representing up to 2.22% (210) of all malaria cases in 2015 [2, 7]. Conversely, this situation is also ideal for the potential spread of artemisinin resistant falciparum strains from SEA to Africa.

Our Vietnamese patient returning from Angola [Chapter 8] with severe malaria presented a slow response (above 100 h) to ACTs, and initially multiple genotypes (later corrected) were found in the environment of the wild type K13-propeller gene. The lack of pharmacokinetic analysis and *in vitro* testing prevented the confirmation of artemisinin resistance, even though early treatment failure was reported. Concomitant to our case report, one 28-day *in-vivo* drug efficacy study from Angola (Zaire province) confirmed very high efficacy of DHA-PPQ (100% ACPR) in children under 5 years of age [125]. Subsequent to our report, the CDC initiated a sentinel site in Luanda province to monitor the *in-vitro and in-vivo* efficacy of DHA-PQ (including K13 mutations) and other ACTs (Plucinsky, *unpublished data*).

There are several potential explanations why artemisinin resistance has developed later in Africa than in SEA, such as a lower selective pressure due to artemisinin derivatives having been introduced more recently in Africa as compared to SAE, and a lower rate of infective malaria being treated with ACTs [126-128]. In addition, absence of a favorable genetic background may have led to the lack of selection of K13 mutations in Africa to date [129].

Even though most of the studies investigating delayed parasite clearance after ACTs treatment in Africa did not report association with K13 mutations [98, 106, 109, 130-

134], a recent article from Lu et al., reports a case of *P. falciparum* artemisinin-resistance in a Chinese patient infected in Equatorial Guinea with a confirmed African strain [135]. In addition, another study found no relationship between K13 mutations and treatment failure [125] and Ugandan children with severe malaria showed a prolonged parasite clearance and half-life in infections with parasites harboring the SNP A578S [136]. Computational modelling using the beta-propeller domain of the *btb-kelch* protein *krp1* suggested that the A578S mutation would induce a structural change with a potentially disruptive effect on normal K13 function [111]. In addition, the LTF after treatment with artemether – lumefantrine has been reported in 4 four patients after travelling to Africa, with no patients containing K13 mutations [137]. Another study in Uganda in 2012–14 showed a low rate of crude ACPR (60.5% and 56.2%, respectively) in *P. falciparum* infected patients (60.5% in retreatment ACT and 56.2% in alternate ACT groups, respectively) [138]. It has also been suggested that three days of ACT treatment is insufficient to clean all parasite genotypes [139, 140]. Therefore, besides the fear of immigration of resistant parasites, such parasites might develop independently in Africa and the adaptive genotype might develop using a different mechanism with parasites in SEA [137]. Thus, even though the presence of K13 mutations and artemisinin resistance seems still negligible in Sub-Saharan Africa, further and continuous investigations should monitor not only K13 mutations but also other mutations associated with the partner drugs.

Our case report raised awareness on the importance of strengthening collaborations

among GMS countries, between the NMCP, the Ministry of Health and other ministries (*i.e.* transport, international cooperation, foreign affairs, etc.) to better track human population movements and prevent malaria among overseas workers [2, 7, 141].

In the current scenario, where both *P.vivax* resistance to chloroquine and *P. falciparum* resistance to artemisinin and piperazine has been confirmed in Vietnam [97, 98, 142], containment efforts need to be prioritized while alternatives to ACTs are developed [143]. On the other hand, malaria therapeutic efficacy studies that have clearly been useful for monitoring resistance during moderate and high transmission intensity are becoming difficult to sustain as malaria transmission decreases, given the paucity of infected patients. More efficient methods that can be sustained at country level and that are able to monitor resistance in all malaria endemic areas in Vietnam, such as molecular surveillance tools, should be implemented and would be useful for supporting containment strategies and advancing malaria elimination in Vietnam.

Conclusion and perspectives

Microscopy and RDTs continue to be very useful tools for the diagnosis of malaria infections in Vietnam, while increased quality control of slide reading and RDT performance, especially at the community level, should be regularly performed following the recommendation of WHO/FIND to ensure the quality of diagnosis and prompt treatment. However, as malaria transmission continues to be reduced and the proportion of asymptomatic and submicroscopic infections is increasing, more

sensitive diagnostic tools should be incorporated in active detection campaigns to detect all malaria infections. PCR-based techniques are sensitive tools for detecting malaria infections with low parasite density; however, their use outside research projects and laboratory centers is still unfeasible due to the lack of infrastructure in most malaria endemic provinces. Thus, field PCR techniques such as the LAMP should urgently be developed and validated as a highly sensitive, cost effective and user friendly tool for the accurate detection of all malaria infections in the remaining endemic and remote areas of Vietnam.

In addition, our study has shown that despite the decrease of malaria transmission in Vietnam, genetic diversity of *P.vivax* parasites is still high. Surveillance of parasite populations including drug resistance markers should become part of malaria molecular surveillance system implemented in sentinel sites to inform treatment policies and elimination strategies. This work has contributed to the understanding of malaria epidemiology in Central Vietnam, and to the development and implementation of molecular tools that will support control and elimination strategies in Vietnam. Conclusions could be useful and extrapolated to other countries in the GMS to support the regional elimination of malaria.

In the near future, malaria might remain a significant health problem in Vietnam due to the development of resistance, imported malaria cases and the absence of alternative affordable antimalarial drugs. In order to shift from control to elimination in the whole country, besides securing the necessary funds, Vietnam needs more

research to understand the contribution of submicroscopic infections to malaria transmission, test new antimalarial drugs, validate field PCR techniques, and identify new cost effective elimination strategies. A good malaria surveillance system, supported by molecular tools, is essential for accelerating malaria elimination in Vietnam.

References

1. Ministry of Health, National Institute of Malariology, Parasitology and Entomology (NIMPE), Hanoi, Vietnam: Guidelines for malaria treatment and diagnosis. *Ministry of Health, Vietnam 2013, number 3232/QD- BYT date 30/8/2013 (Vietnamese version)* 2013.
2. National Malaria Control Program (NMCP): Annual Malaria Report 2014. Hanoi, Vietnam: National Institute of Malariology, Parasitology and Entomology (NIMPE); 2015. Internal report.
3. National Malaria Control Program (NMCP): Annual Malaria Report 2013. Hanoi, Vietnam: National Institute of Malariology, Parasitology and Entomology (NIMPE); 2013. Internal report.
4. National Institute of Malariology, Parasitology and Entomology (NIMPE), Hanoi, Vietnam: Plan for malaria surveillance 7 provinces follow socioeconomic areas. *Ministry of Health Number 2239/QD- BYT date 20/6/2014* 2014. Internal report
5. NIMPE. Molecular Biology Department: Report on PCR results of samples collected in malaria surveillance in 7 provinces follow socioeconomic areas. 2014. Internal report.
6. NIMPE. Molecular Biology Department: Report on PCR results of samples collected in malaria surveillance in 7 provinces follow socioeconomic areas 2015. Internal report.
7. National Malaria Control Program (NMCP): Annual malaria report in 2015 and malaria plan in 2016. pp. 81: National Institute of Malariology Parasitology, Entomology, Hanoi, Vietnam. Internal report 2016:81.
8. Vietnam Bureau of accreditation: Report of Vietnam laboratory accreditation scheme at NIMPE on 23- 24/11/2015 (005Y -T). 2015.
9. National Institute of Malariology Parasitology and Entomology (NIMPE), Hanoi, Vietnam: Plan to build laboratory follow ISO standarts *Number 808/QD- VSR date 07/10/2013 Internal plan* 2013.

10. World Health Organization (WHO): Status report on artemisinin resistance
 January 2014. Available from:
<http://www.who.int/malaria/publications/atoz/updateartemisininresistanceapr2012/en/>. Last access on 30th March, 2016 2014.
11. The global Fun project: Transitional Funding Mechanism (TFM) single country
 applicant: section 1-2 (31 March, 2012, 12 Noon CET). Available from:
<http://www.theglobalfund.org/en/portfolio/country/?loc=VNM>. Last access on
 30th March, 2016. 2012.
12. National Institute of Malariology Parasitology, Entomology (NIMPE), Hanoi,
 Vietnam: Global Fun project 2014 - 2018. Internal plan.
13. Thanh PV, Hong NV, Van Van N, Van Malderen C, Obsomer V, Rosanas-Urgell A,
 Grietens KP, Xa NX, Bancone G, Chowwiwat N, et al: Epidemiology of forest
 malaria in Central Vietnam: the hidden parasite reservoir. *Malar J* 2015, 14:86.
14. Nguyen Quang Thieu LKT, Nguyen Manh Hung, Nguyen Thi Huong Binh: Use of
 PCR assay to assess the prevalence of malaria parasite infection in a high malaria
 endemic area of Quang Tri province. *Journal of malaria and Parasite diseases
 control* – Internal report 2015, 2:9.
15. Le Duc Dao BQP, Nguyen Duc Giang, Ha Viet Vien, Truong Van Hanh, Nguyen
 Van Tuan, Nguyen Dinh yen, Vu Thanh Duc, Phan Thi Thuan: The study on the
 composition of human malaria parasite in Quang Binh province by using nested
 PCR *Journal of malaria and Parasite diseases control* – Internal report 2006, 1:6.
16. Bui Quang Phuc NTT, Le Duc Dao, Nguyen Van Tuan, Truong Van Hanh, Nguyen
 Duc Giang: Species composition of malariae parasite in some high endemic
 malaria areas by nested PCR. *Journal of malaria and Parasite diseases control* –
 Internal report 2011, 4:7.
17. Hoang Cao Sa NVC, Cao Ba Loi: Species composition of malaria parasites and
 drug resistance of *Plasmodium falciparum* in some coastal plain provinces of
 southern Vietnam. *Journal of malaria and Parasite diseases control* – Internal
 report 2015, 3:7.

18. Truong Van Hanh TTD, Nguyen Thi Huong Binh, Nguyen Thi Tra, Nguyen Thi Thu Hien: Identify structure of malaria parasite and proportion of the patients carrying *Plasmodium falciparum* gametocytes in highly transmitted area of Binh phuoc province by PCR methods. *Journal of malaria and Parasite diseases control* – Internal report 2015, 4 ISSN 0868-3735:8.
19. Hung NM: Situation of malaria control in Vietnam during five year (2006 - 2010). *Scientific works of NIMPE: 38th National Malariology and Parasitology conference , part 1, pp.9 – 14*. Hanoi, Vietnam Medical publisher. Number: 240-2011/CXB/28-20/YH; 2011. Internal Report.
20. National Malaria Control Program (NMCP): Annual malaria report in 2012 Hanoi Vietnam 2013. Internal report.
21. Steenkeste N, Rogers WO, Okell L, Jeanne I, Incardona S, Duval L, Chy S, Hewitt S, Chou M, Socheat D, et al: Sub-microscopic malaria cases and mixed malaria infection in a remote area of high malaria endemicity in Rattanakiri province, Cambodia: implication for malaria elimination. *Malar J* 2010, 9:108.
22. Zhou M, Liu Q, Wongsrichanalai C, Suwonkerd W, Panart K, Prajakwong S, Pensiri A, Kimura M, Matsuoka H, Ferreira MU, et al: High prevalence of *Plasmodium malariae* and *Plasmodium ovale* in malaria patients along the Thai-Myanmar border, as revealed by acridine orange staining and PCR-based diagnoses. *Trop Med Int Health* 1998, 3:304-312.
23. Snounou G, White NJ: The co-existence of *Plasmodium*: sidelights from falciparum and vivax malaria in Thailand. *Trends Parasitol* 2004, 20:333-339.
24. Ngo CT, Dubois G, Sinou V, Parzy D, Le HQ, Harbach RE, Manguin S: Diversity of *Anopheles* mosquitoes in Binh Phuoc and Dak Nong Provinces of Vietnam and their relation to disease. *Parasit Vectors* 2014, 7:316.
25. Le Thanh Dong NVK, Mai Anh Loi: Assessment of poperation situation of microscope facilities in southern region- Lam Dong Hanoi, Vietnam: Medical publisher. ISBN: 978-604-66-0950-6; 2015. Internal report.

26. Tran Dinh Dao HNT, Nguyen Hong Phuc: Assesemnt of operation situtation of microscope falcilities in malaria diagnosis in Yen Bai province. *Journal of malaria and Parasite diseases control* – Internal report 2011, 5:80-85.
27. Bui Quang Phuc TTT: The sensitivity, specificity of malaria rapid diagnostic test for detecting *Plasmodium falciparum* and *Plasmodium vivax*. *Journal of malaria and Parasite diseases control* – Internal report 2015, 1:7.
28. Ta Thi Tinh NTH, Truong Thanh Ba, Nguyen Kim Thanh, Le Khanh Thuan: Evaluation and comparation the sensitivity and sensitivity of malaria rapid diagnostic test (Paracheck F test) and light microscopy in detecting *Plasmodium falciparum*. *Journal of malaria and Parasite diseases control* – Internal report 2008, 1:6.
29. PATH: Advancing highly sensitive point-of-care testsfor malaria. Available from from: http://sites.path.org/dx/files/2012/11/PATH-DIAMETER-fact-sheet_April-2017.pdf. Last accessed 9th June 2017. 2017.
30. Alere Inc: Alere Launches the Alere™ Malaria Ag P.f, the First-Ever Rapid Test to Screen Malaria Infection in Asymptomatic Individuals. Available from: <http://www.prnewswire.com/news-releases/alere-launches-the-alere-malaria-ag-pf-the-first-ever-rapid-test-to-screen-malaria-infection-in-asymptomatic-individuals-300444873.html>. Last accessed 6th June, 2017. 2017.
31. Baker J, Ho MF, Pelecanos A, Gatton M, Chen N, Abdullah S, Albertini A, Arie F, Barnwell J, Bell D, et al: Global sequence variation in the histidine-rich proteins 2 and 3 of *Plasmodium falciparum*: implications for the performance of malaria rapid diagnostic tests. *Malar J* 2010, 9:129.
32. Li P, Xing H, Zhao Z, Yang Z, Cao Y, Li W, Yan G, Sattabongkot J, Cui L, Fan Q: Genetic diversity of *Plasmodium falciparum* histidine-rich protein 2 in the China-Myanmar border area. *Acta Trop* 2015, 152:26-31.
33. Lindblade KA, Steinhardt L, Samuels A, Kachur SP, Slutsker L: The silent threat: asymptomatic parasitemia and malaria transmission. *Expert Rev Anti Infect Ther* 2013, 11:623-639.

34. Cheng Q, Cunningham J, Gatton ML: Systematic review of sub-microscopic *P. vivax* infections: prevalence and determining factors. *PLoS Negl Trop Dis* 2015, 9:e3413.
35. Imwong M, Nguyen TN, Tripura R, Peto TJ, Lee SJ, Lwin KM, Suangkanarat P, Jeeyapant A, Vihokhern B, Wongsan K, et al: The epidemiology of subclinical malaria infections in South-East Asia: findings from cross-sectional surveys in Thailand-Myanmar border areas, Cambodia, and Vietnam. *Malar J* 2015, 14:381.
36. Okell LC, Bousema T, Griffin JT, Ouedraogo AL, Ghani AC, Drakeley CJ: Factors determining the occurrence of submicroscopic malaria infections and their relevance for control. *Nat Commun* 2012, 3:1237.
37. Schneider P, Reece SE, van Schaijk BC, Bousema T, Lanke KH, Meaden CS, Gadalla A, Ranford-Cartwright LC, Babiker HA: Quantification of female and male *Plasmodium falciparum* gametocytes by reverse transcriptase quantitative PCR. *Mol Biochem Parasitol* 2015, 199:29-33.
38. Mwingira F, Genton B, Kabanyanyi AN, Felger I: Comparison of detection methods to estimate asexual *Plasmodium falciparum* parasite prevalence and gametocyte carriage in a community survey in Tanzania. *Malar J* 2014, 13:433.
39. Shekalaghe SA, Bousema JT, Kunei KK, Lushino P, Masokoto A, Wolters LR, Mwakalinga S, Mosha FW, Sauerwein RW, Drakeley CJ: Submicroscopic *Plasmodium falciparum* gametocyte carriage is common in an area of low and seasonal transmission in Tanzania. *Trop Med Int Health* 2007, 12:547-553.
40. Schneider P, Bousema JT, Gouagna LC, Otieno S, van de Vegte-Bolmer M, Omar SA, Sauerwein RW: Submicroscopic *Plasmodium falciparum* gametocyte densities frequently result in mosquito infection. *Am J Trop Med Hyg* 2007, 76:470-474.
41. Vallejo AF, Garcia J, Amado-Garavito AB, Arevalo-Herrera M, Herrera S: *Plasmodium vivax* gametocyte infectivity in sub-microscopic infections. *Malar J* 2016, 15:48.

42. Abdul-Ghani R: Towards rapid genotyping of resistant malaria parasites: could loop-mediated isothermal amplification be the solution? *Malar J* 2014, 13:237.
43. Cook J, Aydin-Schmidt B, Gonzalez IJ, Bell D, Edlund E, Nassor MH, Msellem M, Ali A, Abass AK, Martensson A, Bjorkman A: Loop-mediated isothermal amplification (LAMP) for point-of-care detection of asymptomatic low-density malaria parasite carriers in Zanzibar. *Malar J* 2015, 14:43.
44. Morris U, Khamis M, Aydin-Schmidt B, Abass AK, Msellem MI, Nassor MH, Gonzalez IJ, Martensson A, Ali AS, Bjorkman A, Cook J: Field deployment of loop-mediated isothermal amplification for centralized mass-screening of asymptomatic malaria in Zanzibar: a pre-elimination setting. *Malar J* 2015, 14:205.
45. Maeno Y, Culleton R, Quang NT, Kawai S, Marchand RP, Nakazawa S: *Plasmodium knowlesi* and human malaria parasites in Khan Phu, Vietnam: Gametocyte production in humans and frequent co-infection of mosquitoes. *Parasitology* 2016:1-9.
46. Marchand RP, Culleton R, Maeno Y, Quang NT, Nakazawa S: Co-infections of *Plasmodium knowlesi*, *P. falciparum*, and *P. vivax* among Humans and *Anopheles dirus* Mosquitoes, Southern Vietnam. *Emerg Infect Dis* 2011, 17:1232-1239.
47. Maeno Y, Quang NT, Culleton R, Kawai S, Masuda G, Nakazawa S, Marchand RP: Humans frequently exposed to a range of non-human primate malaria parasite species through the bites of *Anopheles dirus* mosquitoes in South-central Vietnam. *Parasit Vectors* 2015, 8:376.
48. Le Thanh Dong TNH, Do Hung Son, Phung Duc Thuan, Vu Thi Hai An, Tran Thi Xuyen, Do Thi Phuong Linh, Pham Nguyen Thuy Vy, Nguyen Thi Minh Chau, Hoang Mai Anh, Nguyen Van Hong, Ngo Viet Thanh, Nguyen Thuy Nha Ca, Phi Ngoc Tru: The first report on malaria parasite in monkeys in Binh Phuoc province. Scientific works of IMPE Ho Chi Minh 1977-2012, part 2, pp 100-105. Internal report. 2011.

49. Jeremiah S, Janagond AB, Parija SC: Challenges in diagnosis of *Plasmodium knowlesi* infections. *Trop Parasitol* 2014, 4:25-30.
50. Rubio JM, Post RJ, van Leeuwen WM, Henry MC, Lindergard G, Hommel M: Alternative polymerase chain reaction method to identify *Plasmodium* species in human blood samples: the semi-nested multiplex malaria PCR (SnM-PCR). *Trans R Soc Trop Med Hyg* 2002, 96 Suppl 1:S199-204.
51. Palaeya V, Lau YL, Mahmud R, Chen Y, Fong MY: Cloning, expression, and immunocharacterization of surface protein containing an altered thrombospondin repeat domain (SPATR) from *Plasmodium knowlesi*. *Malar J* 2013, 12:182.
52. Lau YL, Fong MY, Mahmud R, Chang PY, Palaeya V, Cheong FW, Chin LC, Anthony CN, Al-Mekhlafi AM, Chen Y: Specific, sensitive and rapid detection of human *plasmodium knowlesi* infection by loop-mediated isothermal amplification (LAMP) in blood samples. *Malar J* 2011, 10:197.
53. Jiang N, Chang Q, Sun X, Lu H, Yin J, Zhang Z, Wahlgren M, Chen Q: Co-infections with *Plasmodium knowlesi* and other malaria parasites, Myanmar. *Emerg Infect Dis* 2010, 16:1476-1478.
54. Putaporntip C, Hongsriruang T, Seethamchai S, Kobasa T, Limkittikul K, Cui L, Jongwutiwes S: Differential prevalence of Plasmodium infections and cryptic *Plasmodium knowlesi* malaria in humans in Thailand. *J Infect Dis* 2009, 199:1143-1150.
55. Luchavez J, Espino F, Curameng P, Espina R, Bell D, Chiodini P, Nolder D, Sutherland C, Lee KS, Singh B: Human Infections with *Plasmodium knowlesi*, the Philippines. *Emerg Infect Dis* 2008, 14:811-813.
56. Ng OT, Ooi EE, Lee CC, Lee PJ, Ng LC, Pei SW, Tu TM, Loh JP, Leo YS: Naturally acquired human *Plasmodium knowlesi* infection, Singapore. *Emerg Infect Dis* 2008, 14:814-816.

57. Figtree M, Lee R, Bain L, Kennedy T, Mackertich S, Urban M, Cheng Q, Hudson BJ: *Plasmodium knowlesi* in human, Indonesian Borneo. *Emerg Infect Dis* 2010, 16:672-674.
58. Singh B, Kim Sung L, Matusop A, Radhakrishnan A, Shamsul SS, Cox-Singh J, Thomas A, Conway DJ: A large focus of naturally acquired *Plasmodium knowlesi* infections in human beings. *Lancet* 2004, 363:1017-1024.
59. Khim N, Siv S, Kim S, Mueller T, Fleischmann E, Singh B, Divis PC, Steenkeste N, Duval L, Bouchier C, et al: *Plasmodium knowlesi* infection in humans, Cambodia, 2007-2010. *Emerg Infect Dis* 2011, 17:1900-1902.
60. Olliaro PL, Barnwell JW, Barry A, Mendis K, Mueller I, Reeder JC, Shanks GD, Snounou G, Wongsrichanalai C: Implications of *Plasmodium vivax* Biology for Control, Elimination, and Research. *Am J Trop Med Hyg* 2016, 95:4-14.
61. Imwong M, Snounou G, Pukrittayakamee S, Tanomsing N, Kim JR, Nandy A, Guthmann JP, Nosten F, Carlton J, Looareesuwan S, et al: Relapses of *Plasmodium vivax* infection usually result from activation of heterologous hypnozoites. *J Infect Dis* 2007, 195:927-933.
62. mal, E. R. A. Consultative Group on Basic Science Enabling, Technologies: A research agenda for malaria eradication: basic science and enabling technologies. *PLoS Med* 2011, 8:e1000399.
63. Arnott A, Barry AE, Reeder JC: Understanding the population genetics of *Plasmodium vivax* is essential for malaria control and elimination. *Malar J* 2012, 11:14.
64. Carlton JM, Volkman SK, Uplekar S, Hupalo DN, Pereira Alves JM, Cui L, Donnelly M, Roos DS, Harb OS, Acosta M, et al: Population Genetics, Evolutionary Genomics, and Genome-Wide Studies of Malaria: A View Across the International Centers of Excellence for Malaria Research. *Am J Trop Med Hyg* 2015, 93:87-98.
65. Delgado-Ratto C, Soto-Calle VE, Van den Eede P, Gamboa D, Rosas A, Abatih EN, Rodriguez Ferrucci H, Llanos-Cuentas A, Van Geertruyden JP, Erhart A,

- D'Alessandro U: Population structure and spatio-temporal transmission dynamics of *Plasmodium vivax* after radical cure treatment in a rural village of the Peruvian Amazon. *Malar J* 2014, 13:8.
66. Koepfli C, Rodrigues PT, Antao T, Orjuela-Sanchez P, Van den Eede P, Gamboa D, van Hong N, Bendezu J, Erhart A, Barnadas C, et al: *Plasmodium vivax* Diversity and Population Structure across Four Continents. *PLoS Negl Trop Dis* 2015, 9:e0003872.
 67. Neafsey DE, Galinsky K, Jiang RH, Young L, Sykes SM, Saif S, Gujja S, Goldberg JM, Young S, Zeng Q, et al: The malaria parasite *Plasmodium vivax* exhibits greater genetic diversity than *Plasmodium falciparum*. *Nat Genet* 2012, 44:1046-1050.
 68. Van den Eede P, Erhart A, Van der Auwera G, Van Overmeir C, Thang ND, Hung le X, Anne J, D'Alessandro U: High complexity of *Plasmodium vivax* infections in symptomatic patients from a rural community in central Vietnam detected by microsatellite genotyping. *Am J Trop Med Hyg* 2010, 82:223-227.
 69. Schousboe ML, Ranjitkar S, Rajakaruna RS, Amerasinghe PH, Konradsen F, Morales F, Ord R, Pearce R, Leslie T, Rowland M, et al: Global and local genetic diversity at two microsatellite loci in *Plasmodium vivax* parasites from Asia, Africa and South America. *Malar J* 2014, 13:392.
 70. Anderson TJ, Haubold B, Williams JT, Estrada-Franco JG, Richardson L, Mollinedo R, Bockarie M, Mokili J, Mharakurwa S, French N, et al: Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium falciparum*. *Mol Biol Evol* 2000, 17:1467-1482.
 71. Mu J, Awadalla P, Duan J, McGee KM, Joy DA, McVean GA, Su XZ: Recombination hotspots and population structure in *Plasmodium falciparum*. *PLoS Biol* 2005, 3:e335.
 72. Havryliuk T, Ferreira MU: A closer look at multiple-clone *Plasmodium vivax* infections: detection methods, prevalence and consequences. *Mem Inst Oswaldo Cruz* 2009, 104:67-73.

73. Ferreira MU, Karunaweera ND, da Silva-Nunes M, da Silva NS, Wirth DF, Hartl DL: Population structure and transmission dynamics of *Plasmodium vivax* in rural Amazonia. *J Infect Dis* 2007, 195:1218-1226.
74. Prajapati, S. K.Verma, A.Adak, T.Yadav, R. S.Kumar, A.Eapen, A.Das, M. K.Singh, N.Sharma, S. K.Rizvi, M.A.Dash, A. P.Joshi, H: Supporting the control goal of a malaria free Asia Pacific. <http://www.rollbackmalaria.org/files/files/resources/APMENP%26l.pdf>. 2014.
75. World Health Organization (WHO): World Malaria Report 2015. Available from: <http://www.who.int/malaria/publications/world-malaria-report-2015/en/>. Last accessed 9th Dec, 2015. Geneva 2015.
76. Prajapati SK, Verma A, Adak T, Yadav RS, Kumar A, Eapen A, Das MK, Singh N, Sharma SK, Rizvi MA, et al: Allelic dimorphism of *Plasmodium vivax* gam-1 in the Indian subcontinent. *Malar J* 2006, 5:90.
77. Lin JT, Juliano JJ, Kharabora O, Sem R, Lin FC, Muth S, Menard D, Wongsrichanalai C, Rogers WO, Meshnick SR: Individual *Plasmodium vivax* msp1 variants within polyclonal *P. vivax* infections display different propensities for relapse. *J Clin Microbiol* 2012, 50:1449-1451.
78. Daniels RF, Rice BL, Daniels NM, Volkman SK, Hartl DL: The utility of genomic data for *Plasmodium vivax* population surveillance. *Pathog Glob Health* 2015, 109:153-161.
79. White NJ: Determinants of relapse periodicity in *Plasmodium vivax* malaria. *Malar J* 2011, 10:297.
80. Robinson LJ, Wampfler R, Betuela I, Karl S, White MT, Li Wai Suen CS, Hofmann NE, Kinboro B, Waltmann A, Brewster J, et al: Strategies for understanding and reducing the *Plasmodium vivax* and *Plasmodium ovale* hypnozoite reservoir in Papua New Guinean children: a randomised placebo-controlled trial and mathematical model. *PLoS Med* 2015, 12:e1001891.
81. Betuela I, Rosanas-Urgell A, Kiniboro B, Stanisic DI, Samol L, de Lazzari E, Del Portillo HA, Siba P, Alonso PL, Bassat Q, Mueller I: Relapses contribute

- significantly to the risk of *Plasmodium vivax* infection and disease in Papua New Guinean children 1-5 years of age. *J Infect Dis* 2012, 206:1771-1780.
82. Silachamroon U, Krudsood S, Treeprasertsuk S, Wilairatana P, Chalearmrult K, Mint HY, Maneekan P, White NJ, Gourdeuk VR, Brittenham GM, Looareesuwan S: Clinical trial of oral artesunate with or without high-dose primaquine for the treatment of vivax malaria in Thailand. *Am J Trop Med Hyg* 2003, 69:14-18.
 83. Lin JT, Patel JC, Kharabora O, Sattabongkot J, Muth S, Ubalee R, Schuster AL, Rogers WO, Wongsrichanalai C, Juliano JJ: *Plasmodium vivax* isolates from Cambodia and Thailand show high genetic complexity and distinct patterns of *P. vivax* multidrug resistance gene 1 (*pvmdr1*) polymorphisms. *Am J Trop Med Hyg* 2013, 88:1116-1123.
 84. Restrepo E, Imwong M, Rojas W, Carmona-Fonseca J, Maestre A: High genetic polymorphism of relapsing *P. vivax* isolates in northwest Colombia. *Acta Trop* 2011, 119:23-29.
 85. Chen N, Auliff A, Rieckmann K, Gatton M, Cheng Q: Relapses of *Plasmodium vivax* infection result from clonal hypnozoites activated at predetermined intervals. *J Infect Dis* 2007, 195:934-941.
 86. Kim JR, Nandy A, Maji AK, Addy M, Dondorp AM, Day NP, Pukrittayakamee S, White NJ, Imwong M: Genotyping of *Plasmodium vivax* reveals both short and long latency relapse patterns in Kolkata. *PLoS One* 2012, 7:e39645.
 87. World Health Organization (WHO): Guideline for the treatment of Malaria. Available from: <http://www.who.int/malaria/publications/atoz/9789241549127/en/>. Last accessed 12thDec, 2015. 2015.
 88. Baird JK, Hoffman SL: Primaquine therapy for malaria. *Clin Infect Dis* 2004, 39:1336-1345.
 89. Koepfli C, Schoepflin S, Bretscher M, Lin E, Kiniboro B, Zimmerman PA, Siba P, Smith TA, Mueller I, Felger I: How much remains undetected? Probability of molecular detection of human Plasmodia in the field. *PLoS One* 2011, 6:e19010.

90. Sutton PL: A call to arms: on refining *Plasmodium vivax* microsatellite marker panels for comparing global diversity. *Malar J* 2013, 12:447.
91. Barry AE, Waltmann A, Koepfli C, Barnadas C, Mueller I: Uncovering the transmission dynamics of *Plasmodium vivax* using population genetics. *Pathog Glob Health* 2015, 109:142-152.
92. Global Health group, Global Health Science UCSF: Surveillance systems to facilitate malaria elimination. Available from: <http://globalhealthsciences.ucsf.edu/sites/default/files/content/ghg/mei-surveillance-systems-to-facilitate.pdf>. Last access 24 July 2016 January 2014.
93. Branch OH, Takala S, Kariuki S, Nahlen BL, Kolczak M, Hawley W, Lal AA: *Plasmodium falciparum* genotypes, low complexity of infection, and resistance to subsequent malaria in participants in the Asembo Bay Cohort Project. *Infect Immun* 2001, 69:7783-7792.
94. Thanh NV, Toan TQ, Cowman AF, Casey GJ, Phuc BQ, Tien NT, Hung NM, Biggs BA: Monitoring for *Plasmodium falciparum* drug resistance to artemisinin and artesunate in Binh Phuoc Province, Vietnam: 1998-2009. *Malar J* 2010, 9:181.
95. Thanh NV, Thuy-Nhien N, Tuyen NT, Tong NT, Nha-Ca NT, Dong LT, Quang HH, Farrar J, Thwaites G, White NJ, et al: Rapid decline in the susceptibility of *Plasmodium falciparum* to dihydroartemisinin-piperazine in the south of Vietnam. *Malar J* 2017, 16:27.
96. National Institute of Malaria, Parasitology and Entomology (NIMPE), Hanoi, Vietnam: Review of antimalaria drug efficacy over the last 10 years in Vietnam. NIMPE report to WHO. NIMPE Hanoi. Vietnam. 2012. Internal Report.
97. World Health Organization (WHO): Status report on artemisinin and ACT resistance. Available from: <http://www.who.int/malaria/publications/atoz/status-rep-artemisinin-resistance-sep2014.pdf>. Last accessed 7thDec, 2015. 2015.
98. World Health Organization (WHO): Artemisinin and artemisinin -based combination therapy resistance. Available from:

<http://apps.who.int/iris/bitstream/10665/250294/1/WHO-HTM-GMP-2016.11-eng.pdf>. Last accessed 26th Oct, 2016. 2016.

99. Truong Van Hanh NTHB, Tran Thanh Duong, Nguyen Thi Tra, Bui Quang Phuc, Do Manh Ha: Prevalence of K13- propeller polymorphism in *Plasmodium falciparum* isolates from Binh Phuoc province. *Journal of malaria and Parasite diseases control* – Internal report 2015, 6:6.
100. Nguyen Van Tuan HDt, Bui Quang Phuc , Vu Duc Chinh, Yoshimasa Meano, Nguyen Thi Huong Binh: The richness of malaria vectors and mutation of the K13 gene of artemisinin - resistant *Plasmodium falciparum* in Binh Phuoc and Dak Nong provinces, 2010-2014. *Journal of malaria and Parasite diseases control* – Internal report 2015, 5:11.
101. Thuy-Nhien N, Tuyen NK, Tong NT, Vy T, Thanh NV, Van HT, Huong-Thu P, Quang HH, Boni MF, Dolecek C, et al: K13-Propeller Mutations in *Plasmodium falciparum* Populations in Malaria Endemic Regions of Vietnam from 2009 to 2016. *Antimicrob Agents Chemother* 2017.
102. Spring MD, Lin JT, Manning JE, Vanachayangkul P, Somethy S, Bun R, Se Y, Chann S, Ittiverakul M, Sia-ngam P, et al: Dihydroartemisinin-piperaquine failure associated with a triple mutant including kelch13 C580Y in Cambodia: an observational cohort study. *Lancet Infect Dis* 2015, 15:683-691.
103. Ministry of Health, National Institute of Malariology, Parasitology and Entomology (NIMPE), Hanoi, Vietnam: Guideline for malaria diagnosis and treatment. *Ministry of Health, decision number 4605/QD- BYT date 24/11/2009 (Vietnamese version)* 2009.
104. Ministry of Health, National Institute of Malariology, Parasitology and Entomology (NIMPE): Guidelines for malaria diagnosis and treatment. *Ministry of Health, Hanoi, Vietnam number 339/QD- BYT date 31/01/2007 (Vietnamese version)* 2007.

105. Miotto O, Almagro-Garcia J, Manske M, Macinnis B, Campino S, Rockett KA, Amaratunga C, Lim P, Suon S, Sreng S, et al: Multiple populations of artemisinin-resistant *Plasmodium falciparum* in Cambodia. *Nat Genet* 2013, 45:648-655.
106. Ashley EA, Dhorda M, Fairhurst RM, Amaratunga C, Lim P, Suon S, Sreng S, Anderson JM, Mao S, Sam B, et al: Spread of artemisinin resistance in *Plasmodium falciparum* malaria. *N Engl J Med* 2014, 371:411-423.
107. Tun KM, Imwong M, Lwin KM, Win AA, Hlaing TM, Hlaing T, Lin K, Kyaw MP, Plewes K, Faiz MA, et al: Spread of artemisinin-resistant *Plasmodium falciparum* in Myanmar: a cross-sectional survey of the K13 molecular marker. *Lancet Infect Dis* 2015, 15:415-421.
108. Wang Z, Shrestha S, Li X, Miao J, Yuan L, Cabrera M, Grube C, Yang Z, Cui L: Prevalence of K13-propeller polymorphisms in *Plasmodium falciparum* from China-Myanmar border in 2007-2012. *Malar J* 2015, 14:168.
109. Menard D, Khim N, Beghain J, Adegnika AA, Shafiul-Alam M, Amodu O, Rahim-Awab G, Barnadas C, Berry A, Boum Y, et al: A Worldwide Map of *Plasmodium falciparum* K13-Propeller Polymorphisms. *N Engl J Med* 2016, 374:2453-2464.
110. Torrentino-Madamet M, Fall B, Benoit N, Camara C, Amalvict R, Fall M, Dionne P, Ba Fall K, Nakoulima A, Diatta B, et al: Limited polymorphisms in k13 gene in *Plasmodium falciparum* isolates from Dakar, Senegal in 2012-2013. *Malar J* 2014, 13:472.
111. Mohon AN, Alam MS, Bayih AG, Folefoc A, Shahinas D, Haque R, Pillai DR: Mutations in *Plasmodium falciparum* K13 propeller gene from Bangladesh (2009-2013). *Malar J* 2014, 13:431.
112. Lin SG LD, Zhuo KR et al.: Determination of sensitivity of *Plasmodium falciparum* to antimalarials in Ledong County, Hainan Province. *China Tropical Medicine* 5, 1707-1708 2005
113. Zhang KY, Zhou JX, Wu Z, Huang QL: [Susceptibility of *Plasmodium falciparum* to chloroquine, piperazine, amodiaquine, mefloquine and quinine with in vitro

- microtechnique in Hainan Island]. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi* 1987, 5:165-169.
114. Phuc BQ: Efficacy and safety of dihydroartemisin-piperaquine for the treatment of uncomplicated *Plasmodium falciparum* malaria in Vietnam in 2014. NIMPE Report to WHO on routine monitoring sentinel sites. NIMPE, Hanoi. Vietnam 2015.
 115. NIMPE. Department of Malaria treatment and research: Report on malaria diagnosis and treatment and antimalaria drugs resistance in 2014 - 2015. Report to NMCP, July, 2015 (Vietnamese version). 2015. Internal Report
 116. Amaratunga C, Lim P, Suon S, Sreng S, Mao S, Sopha C, Sam B, Dek D, Try V, Amato R, et al: Dihydroartemisinin-piperaquine resistance in *Plasmodium falciparum* malaria in Cambodia: a multisite prospective cohort study. *Lancet Infect Dis* 2016, 16:357-365.
 117. Duru V, Khim N, Leang R, Kim S, Domergue A, Kloeung N, Ke S, Chy S, Eam R, Khean C, et al: *Plasmodium falciparum* dihydroartemisinin-piperaquine failures in Cambodia are associated with mutant K13 parasites presenting high survival rates in novel piperaquine in vitro assays: retrospective and prospective investigations. *BMC Med* 2015, 13:305.
 118. World Health Organization (WHO): Emergency response to artemisinin resistance in the Greater Mekong subregion. Regional Framework for Action 2013-2015. Available from: <http://www.who.int/malaria/publications/atoz/9789241505321/en/>. Last accessed 7thDec, 2015. 2013.
 119. White NJ: Artemisinin resistance--the clock is ticking. *Lancet* 2010, 376:2051-2052.
 120. Ministry of Health, National Institute of Malariology Parasitology, Entomology (NIMPE), Hanoi, Vietnam: Guidelines for malaria diagnosis and treatment *Ministry of Health, Vietnam 2016, number 4845/QD- BYT date 8/9/2016 (Vietnamese version)* 2016.

121. Nguyen TD, Olliaro P, Dondorp AM, Baird JK, Lam HM, Farrar J, Thwaites GE, White NJ, Boni MF: Optimum population-level use of artemisinin combination therapies: a modelling study. *Lancet Glob Health* 2015, 3:e758-766.
122. The Economic: One among many. China has become big in Africa. Now for the backlash. Available from: <http://www.economist.com/news/middle-east-and-africa/21639554-china-has-become-big-africa-now-backlash-one-among-many>. Last access 29th Jan 2017 2015.
123. Consular department, Ministry of Foreign affairs of Vietnam: Review of Vietnamese migration abroad. Available from: http://eeas.europa.eu/archives/delegations/vietnam/documents/eu_vietnam/vn_migration_abroad_en.pdf. Last accessed 29th Jan 2017. 2012.
124. Liu Y, Zhang HW, Zhou RM, Yang CY, Qian D, Zhao YL, Xu BL: First imported relapse case of *Plasmodium vivax* malaria and analysis of its origin by CSP sequencing in Henan Province, China. *Malar J* 2014, 13:448.
125. Plucinski MM, Talundzic E, Morton L, Dimbu PR, Macaia AP, Fortes F, Goldman I, Lucchi N, Stennies G, MacArthur JR, Udhayakumar V: Efficacy of artemether-lumefantrine and dihydroartemisinin-piperaquine for treatment of uncomplicated malaria in children in Zaire and Uige Provinces, angola. *Antimicrob Agents Chemother* 2015, 59:437-443.
126. World Health Organization (WHO): World malaria report 2014. Available from: http://www.who.int/malaria/publications/world_malaria_report_2014/report/en/. Last accessed 12thDec, 2015 2014.
127. World Health Organization (WHO): Status report on artemisinin resistance. Available from http://www.who.int/malaria/publications/atoz/status_rep_artemisinin_resistance_sep2014.pdf?ua=1. Last access on 15 March 2016. 2014.
128. Mori Y, Notomi T: Loop-mediated isothermal amplification (LAMP): a rapid, accurate, and cost-effective diagnostic method for infectious diseases. *J Infect Chemother* 2009, 15:62-69.

129. Woodrow CJ, White NJ: The clinical impact of artemisinin resistance in Southeast Asia and the potential for future spread. *FEMS Microbiol Rev* 2016.
130. MalariaGEN *Plasmodium falciparum* community project: Genomic epidemiology of the current wave of artemisinin resistance malaria. Available from: <http://biorxiv.org/content/biorxiv/early/2015/05/22/019737.full.pdf>. Last access 2 August 2016. 2015.
131. Miotto O, Amato R, Ashley EA, MacInnis B, Almagro-Garcia J, Amaratunga C, Lim P, Mead D, Oyola SO, Dhorda M, et al: Genetic architecture of artemisinin-resistant *Plasmodium falciparum*. *Nat Genet* 2015, 47:226-234.
132. Conrad MD, Bigira V, Kapisi J, Muhindo M, Kanya MR, Havlir DV, Dorsey G, Rosenthal PJ: Polymorphisms in K13 and falcipain-2 associated with artemisinin resistance are not prevalent in *Plasmodium falciparum* isolated from Ugandan children. *PLoS One* 2014, 9:e105690.
133. Kamau E, Campino S, Amenga-Etego L, Drury E, Ishengoma D, Johnson K, Mumba D, Kekre M, Yavo W, Mead D, et al: K13-propeller polymorphisms in *Plasmodium falciparum* parasites from sub-Saharan Africa. *J Infect Dis* 2015, 211:1352-1355.
134. Taylor SM, Parobek CM, DeConti DK, Kayentao K, Coulibaly SO, Greenwood BM, Tagbor H, Williams J, Bojang K, Njie F, et al: Absence of putative artemisinin resistance mutations among *Plasmodium falciparum* in Sub-Saharan Africa: a molecular epidemiologic study. *J Infect Dis* 2015, 211:680-688.
135. Lu F, Culleton R, Zhang M, Ramaprasad A, von Seidlein L, Zhou H, Zhu G, Tang J, Liu Y, Wang W, et al: Emergence of Indigenous Artemisinin-Resistant *Plasmodium falciparum* in Africa. *N Engl J Med* 2017, 376:991-993.
136. Hawkes M, Conroy AL, Opoka RO, Namasopo S, Zhong K, Liles WC, John CC, Kain KC: Slow Clearance of *Plasmodium falciparum* in Severe Pediatric Malaria, Uganda, 2011-2013. *Emerg Infect Dis* 2015, 21:1237-1239.
137. Sutherland CJ, Lansdell P, Sanders M, Muwanguzi J, van Schalkwyk DA, Kaur H, Nolder D, Tucker J, Bennett HM, Otto TD, et al: Pfk13-independent treatment

- failure in four imported cases of *Plasmodium falciparum* malaria given artemether-lumefantrine in the UK. *Antimicrob Agents Chemother* 2017.
138. Mavoko HM, Nabasumba C, da Luz RI, Tinto H, D'Alessandro U, Kambugu A, Baraka V, Rosanas-Urgell A, Lutumba P, Van Geertruyden JP: Efficacy and safety of re-treatment with the same artemisinin-based combination treatment (ACT) compared with an alternative ACT and quinine plus clindamycin after failure of first-line recommended ACT (QUINACT): a bicentre, open-label, phase 3, randomised controlled trial. *Lancet Glob Health* 2017, 5:e60-e68.
 139. Sutherland CJ: Rescuing artemisinin combination therapy in Africa. *Lancet Glob Health* 2017, 5:e8-e9.
 140. van Schalkwyk DA, Sutherland CJ: Malaria resistance to non-artemisinin partner drugs: how to reACT. *Lancet Infect Dis* 2015, 15:621-623.
 141. National Malaria Control Program (NMCP): Annual Malaria Report during 6 first months of 2015. NIMPE, Hanoi. Vietnam 2015. Internal report.
 142. Pham VT, Hong NV, Nguyen VV, Louisa M, Baird K, Nguyen XX, Grietens KP, Le XH, Tran TD, Rosanas-Urgell A, et al: Confirmed *Plasmodium vivax* resistance to chloroquine in Central Vietnam. *Antimicrob Agents Chemother* 2015.
 143. Kuhen KL, Chatterjee AK, Rottmann M, Gagaring K, Borboa R, Buenviaje J, Chen Z, Francek C, Wu T, Nagle A, et al: KAF156 is an antimalarial clinical candidate with potential for use in prophylaxis, treatment, and prevention of disease transmission. *Antimicrob Agents Chemother* 2014, 58:5060-5067.

Curriculum vitae

Nguyen Van Hong was born in Son la province, Vietnam in 1981. She graduated as a medical doctor in 2005 from the Hanoi Medical University in Vietnam. Immediately afterwards, she started to work at the National Institute of Malariology, Parasitology and Entomology (NIMPE), in Hanoi, Vietnam. She studied for a PhD in the laboratory of the Clinical department and Molecular Biology at NIMPE, Vietnam, and the laboratory of the Malariology Unit, Department of Biomedical Sciences at the Institute of Tropical Medicine in Antwerp, and for the doctoral programme at the Faculty of Medicine and Health Sciences at the University of Antwerp, Belgium. She received a PhD scholarship from the Belgium Government (Framework Agreement III, DGD) to carry out her research. During her PhD, she focused on improving diagnostic methods for malaria.

Publications

1. **Hong NV**, Delgado-Ratto C, Thanh PV, Van den Eede P, Guetens P, Binh NT, Phuc BQ, Duong TT, Van Geertruyden JP, D'Alessandro U, et al: Population Genetics of *Plasmodium vivax* in Four Rural Communities in Central Vietnam. *PLoS Negl Trop Dis* 2016, 10:e0004434.
2. **Van Hong N**, Amambua-Ngwa A, Tuan NQ, Cuong do D, Giang NT, Van Dung N, Tinh TT, Van Tien N, Phuc BQ, Duong TT, et al: Severe Malaria Not Responsive to Artemisinin Derivatives in Man Returning from Angola to Vietnam. *Emerg Infect Dis* 2015, 21:1265.
3. Pham VT, **Hong NV**, Nguyen VV, Louisa M, Baird K, Nguyen XX, Grietens KP, Le XH, Tran TD, Rosanas-Urgell A, et al: Confirmed *Plasmodium vivax* resistance to chloroquine in Central Vietnam. *Antimicrob Agents Chemother* 2015.
4. Thanh PV, **Hong NV**, Van Van N, Van Malderen C, Obsomer V, Rosanas-Urgell A, Grietens KP, Xa NX, Bancone G, Chowwiwat N, et al: Epidemiology of forest malaria in Central Vietnam: the hidden parasite reservoir. *Malar J* 2015, 14:86.
5. Koepfli C, Rodrigues PT, Antao T, Orjuela-Sanchez P, Van den Eede P, Gamboa D, **van Hong N**, Bendezu J, Erhart A, Barnadas C, et al: *Plasmodium vivax* Diversity and Population Structure across Four Continents. *PLoS Negl Trop Dis* 2015, 9:e0003872.
6. Thriemer K, **Hong NV**, Rosanas-Urgell A, Phuc BQ, Ha do M, Pockele E, Guetens P, Van NV, Duong TT, Amambua-Ngwa A, et al: Delayed parasite clearance after treatment with dihydroartemisinin-piperaquine in *Plasmodium falciparum* malaria patients in central Vietnam. *Antimicrob Agents Chemother* 2014, 58:7049-7055.
7. **Van Hong N**, Amambua-Ngwa A, Tuan NQ, Cuong do D, Giang NT, Van Dung N, Tinh TT, Van Tien N, Phuc BQ, Duong TT, et al: Severe malaria not responsive to artemisinin derivatives in man returning from Angola to Vietnam. *Emerg Infect Dis* 2014, 20:1199-1202.

8. **Van Hong N**, van den Eede P, Van Overmeir C, Vythilingam I, Rosanas-Urgell A, Vinh Thanh P, Thang ND, Hung NM, Hung le X, D'Alessandro U, Erhart A: A modified semi-nested multiplex malaria PCR (SnM-PCR) for the identification of the five human *Plasmodium* species occurring in Southeast Asia. *Am J Trop Med Hyg* 2013, 89:721-723.
9. **Nguyen HV**, van den Eede P, van Overmeir C, Thang ND, Hung le X, D'Alessandro U, Erhart A: Marked age-dependent prevalence of symptomatic and patent infections and complexity of distribution of human *Plasmodium* species in central Vietnam. *Am J Trop Med Hyg* 2012, 87:989-995.
10. Speybroeck N, Praet N, Claes F, **Van Hong N**, Torres K, Mao S, Van den Eede P, Thi Thinh T, Gamboa D, Sochantha T, et al: True versus apparent malaria infection prevalence: the contribution of a Bayesian approach. *PLoS One* 2011, 6:e16705.
11. Van den Eede P, Vythilingam I, Ngo DT, **Nguyen VH**, Le XH, D'Alessandro U, Erhart A: *Plasmodium knowlesi* malaria in Vietnam: some clarifications. *Malar J* 2010, 9:20.
12. Van den Eede P, **Van HN**, Van Overmeir C, Vythilingam I, Duc TN, Hung le X, Manh HN, Anne J, D'Alessandro U, Erhart A: Human *Plasmodium knowlesi* infections in young children in central Vietnam. *Malar J* 2009, 8:249.

Other publications

13. **Hong Nguyen Van**, Peter Van den Eede, Chantal Van Overmeir, Indra Vythilingam, Thang Ngo Duc, Le Xuan Hung, Hung Nguyen Manh, Jozef Anné, Umberto D'Alessandro and Annette Erhart. The first report on human *Plasmodium knowlesi* infection in Vietnam. Scientific works of NIMPE 2006 – 2011, part 1, pp.2005 – 2010. Internal Report.
14. Le Thanh Dong, Trinh Ngoc Hai, Do Hung Son, Phung Duc Thuan, Vu Thi Hai An, Tran Thi Xuyen, Do Thi Phuong Linh, Pham Nguyen Thuy Vy, Nguyen Thi Minh Chau, Hoang Mai Anh, Nguyen Van Hong, Ngo Viet Thanh, Nguyen Thuy Nha Ca,

Phi Ngoc Tru. The first report on malaria parasite in monkeys in Binh Phuoc province. Scientific works of IMPE Ho Chi Minh 1977-2012, part 2, pp 100-105. Internal report.

15. **Hong Nguyen Van**, Bui Quang Phuc, Nguyen Thi Thu Hien, Vu Thi Sang. Assessment sensitivity of *Plasmodium falciparum* strains with antimalaria drugs by Elisa technique. *Journal of Malaria and Parasite diseases control*- Internal Report 2013, No2, pp 52-61.
16. Ta Thi Tinh, Do Manh Ha, Nguyen Duc Truong, Vu Van Thai, Nguyen Thi Thuy Duong, Do Minh Tuan, **Nguyen Van Hong**. Efficiency and safety of CV artequick in treatment uncomplicated *Plasmodium falciparum*. *Journal of Malaria and Parasite diseases control* - Internal report 2013, No6, pp 69-81.

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