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"Epidemiology of Plasmodium vivax malaria in Central Vietnam "

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ABSTRACT

Despite the success of malaria control programe in the past decade, malaria remains an important public health problem in Vietnam. A challenge faced by the National Malaria Control and Elimination Programme is the ability to target and eliminate the remaining parasite reservoir in endemic areas The cross-sectional and longitudinal studies was caried out in malaria endemic area in Central, Vietnam and both of them combined PCR and LM for malaria parasite detection methodes. The results of cross-sectional study confirmed the high malaria parasite reservoir detection due to PCR alone and asymptomatic with LM malaria parasite positive with two malaria parasite species contribution to malaria burden in study area which included Plasmodium falciparum and Plasmodium viviax. The longgitudinal study followed up P.vivax infected patients for two years and within first month there were three P.vivax cases ware confirmed resistance Chloroquine. After ward, the high number of P.vivax recurrences with submicroscopic and asymptomatic infection were recoded despire the higdose of PQ treatement with direct observation of study team. To over come asymptomatic and sub-patent reservoir calls for an immediate combination of molecular and LM detecting malaria hotpots for targeted interventions. P.vivax resistance CQ and high morbidity of P.vivax recurrences after PQ treatment are alarmed to NMCP changing treatment policy to new drugs with high effectiveneess and short course.

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EPIDEMIOLOGY OF PLASMODIUM VIVAX MALARIA IN CENTRAL VIETNAM

Pham Vinh Thanh

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Thèse présentée en vue de l'obtention du grade de docteur en Sciences de la santé publique Secteur des sciences de la santé

Summary

Despite the success of malaria control programe in the past decade, malaria remains an important public health problem in Vietnam. A challenge faced by the National Malaria Control and Elimination Programme is the ability to target and eliminate the remaining parasite reservoir in endemic areas

The cross-sectional and longitudinal studies was caried out in malaria endemic area in Central, Vietnam and both of them combined PCR and LM for malaria parasite detection methodes. The results of cross-sectional study confirmed the high malaria parasite reservoir detection due to PCR alone and asymptomatic with LM malaria parasite positive with two malaria parasite species contribution to malaria burden in study area which included *Plasmodium falciparum* and *Plasmodium viviax*. The longgitudinal study followed up *P.vivax* infected patients for two years and within first month there were three *P.vivax* cases ware confirmed resistance Chloroquine. After ward, the high number of *P.vivax* recurrences with submicroscopic and asymptomatic infection were recoded despire the higdose of PQ treatement with direct observation of study team.

To over come asymptomatic and sub-patent reservoir calls for an immediate combination of molecular and LM detecting malaria hotpots for targeted interventions. *P.vivax* resistance CQ and high morbidity of P.vivax recurrences after PQ treatment are alarmed to NMCP changing treatment policy to new drugs with high effectiveneess and short course.

Biography

Pham Vinh Thanh was born in Hanoi city, Vietnam in 1970. He graduated as a medical doctor in 1994 from the Hanoi Medical University in Vietnam. Immediately afterwards,

he started to work at the National Institute of Malariology, Parasitology and Entomology (NIMPE), in Hanoi, Vietnam. In 2007, he graduated master of Public Health at Mahidol University in Thailand. He received a PhD scholarship from Institute of Tropical Medicine in Antwerp, Belgium to carried out his research focusing on epidemiology of *Plasmodium vivax* malaria.

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List of abbreviations

ACD	Active Case Detection
ACPR	Adequate Clinical and Parasitological Response
ACT	Artemisinin-based Combination Therapy
CHC	Commune Health Central
CQ	Chloroquine
PvCQR	Plasmodium vivax Resistance to chloroquine
DOT	Directly Observed
ETF	Early Treatment Failure
FPBS	Filter Paper Blood Samples
G6PD	Glucose-6-Phosphate Dehydrogenase
GF	Global Fund
GIS	Geographic Information System
GMS	Greater Mekong Sub-region
IQR	Interquartile Range
IRS	Indoor Residual Spraying
ITN	Insecticide treated nest
LAMP	Loop-mediated isothermal Amplification
LLIN	Long Lasting Insecticidal Net
LM	Light Microscopy
LTF	Late Treatment Failure
NIMPE	National Institute of Malariology, Parasitology and
	Entomology
NMCP	National Malaria Control Programme
P. falciparum	Plasmodium falciparum
P. knowlesi	Plasmodium knowlesi
P. malariae	Plasmodium malariae
P. ovale	Plasmodium ovale
P. vivax	Plasmodium vivax
PCD	Passive Case Detection
PCR	Polymerase Chain Reaction
РСТ	Parasite clearance time
PQ	Primaquine
RDT	Rapid Diagnostic Test
SEA	Southeast Asia
SnM- PCR	Semi-nested multiplex Malaria PCR

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CHAPTER I General Introduction

1. Rational

Plasmodium vivax (*P. vivax*) is the most worldwide distributed malaria parasite species. In 2015, WHO reported about 8.5 million cases due to *P. vivax* with a majority occurring in South East Asia (58%) [1, 2]. Of the five species of *Plasmodium* that affect humans, *P. vivax* and *P. ovale* species [3] form hypnozoites, which are dormant parasite stages in the liver that cause relapse weeks to years after the primary infection. *P. vivax* preferentially invades reticulocytes, and repeated illness causes chronic anaemia [4]. The major complication is anaemia in young children particularly in Indonesia [5] and in Papua New Guinea [6]. Occasionally, in old patients *P. vivax* can cause severe and complicated malaria similar to *P. falciparum* species [7, 8]. During pregnancy, infection with *P. vivax* increases the risk of abortion and reduces birth weight [9, 10].

Recently, the WHO made a courageous commitment to elimination of malaria in the Greater Mekong area by 2030 [11]. In areas where both *P. vivax* and *P. falciparum* coexist, the incidence of *P. vivax* decreases less rapidly than that of *P. falciparum*. Hence, *P. vivax* persists as the principal cause of malaria in these areas and as the main barrier to elimination of the disease. Indeed, *P. vivax* has several distinct biological characteristics that challenges its control and elimination. *P. vivax* sporozoites develop faster than *P. falciparum* inside infected female mosquitoes at equivalent temperatures, which contributes to its wider geographic range [12]. Hence, many patients have sufficient gametocytaemia to allow transmission before an infection is diagnosed or treated [13-15]. The preference for invading reticulocytes leads to low parasite densities that are more difficult to detect than *P. falciparum*, which invades blood cells of any age [16]. This causes missing diagnosis by routine tools, i.e. LM and RDTs, which have limitated sensitivity to detect low parasite densities. A dormant liver stage, known as a hypnozoite, which can relapse weeks or months after a primary infection [17]. The hypnozoite presents particular challenges for control and elimination of *P. vivax* malaria because it is undetectable using currently available diagnostic methods. It is not susceptible to drugs that target blood-stage forms of the parasite, and the only available drug targeting hypnozoites is primaquine (PQ). However, PQ requires a 14-day treatment following WHO's recommendations. This regimen has significant problems with poor treatment adherence, and the risk of hemolysis in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency [18, 19].

To clear blood-stage forms, chloroquine (CQ) is used against susceptible strains of *P. vivax* and gametocytocidal of this parasite specie and it is not active against intrahepatic forms [20]. However, this drug is challenging to malaria elimination because *P. vivax* parasites are developing resistance to this drug. The first report on *P. vivax* CQ resistance (PvCQR) was in the island of New Guinea in 1989 [21]. More recently, reports reveal declining CQ efficacy against *P. vivax* in Ethiopia, Madagascar, India, western Indonesia, South Korea, Myanmar, Thailand, Brazil and Columbia [22-27].

Despite this burden, malaria decreased dramatically in the last two decades in Vietnam and today the country is on track for more than 75% decrease of malaria incidence rate [28]. By the end of 2011, Vietnam officially endorsed malaria elimination, aiming for a *P. falciparum* malaria elimination in 2025 and malaria free in 2030 [29-31]. This context emphasized that *P. vivax* elimination will be following the success of *P. falciparum* elimination. To date, together with success of malaria control, the proportion of *P. vivax* increased from 20% to 40% of total malaria burden in the country [28, 32, 33]. In 2016, the majority of confirmed *P.vivax* cases (83.54%; 1,462 *P. vivax*/ totals confirmed malaria 1,750) concentrated in ten provinces located in Central Highland and Southeast regions [34].

Current Vietnam malaria control and elimination strategies include improved surveillance, intensified community education, and diversified vector control interventions for high-risk populations [34]. Malaria surveillance is improved by moving to active case detection (ACD) strategy to target the human reservoir: infected with the malaria parasite, but without any malaria clinical symptoms (particularly in *P. vivax* infected patients) [35]. Therefore, loop-mediated isothermal amplification (LAMP) detection tool is under evaluation by the National Institute of Malariology, parasitology and entomology (NIMPE). If successful, LAMP will be useful in ACD campaigns contributing to the success of malaria elimination in Vietnam.

The main difficulty in controlling vivax malaria lies in the need to radically treat not only blood forms but also the dormant hepatic forms (hypnozoites). Vietnamese treatment guidelines currently recommend for radical cure a 3-day course of CQ (total of 25 mg/kg) together with a 14-day course of primaquine (PQ) (0.25 mg/kg/day). Nevertheless, between 2007 and 2009 [36], instead of the 14-day course, PQ was given for 10 days at a higher dose (0.5 mg/kg/day).

Currently, malaria elimination in Vietnam is faced with undetected malaria infections, antimalarial drug resistance, and imported cases. Asymptomatic malaria infections are common in remote and forested areas of Central Vietnam particularly among local ethnic minorities in which the burden of *P*. vivax is particularly high [37-39]. P. falciparum resistance to artemisinins has been already reported in five provinces of Central Vietnam [40, 41]. Moreover, the control of *P. vivax* is another challenge, as this species is becoming increasingly prevalent [2, 33, 37, 42]. However, P. vivax chloroquine resistance (PvCQR) is still rare and only one PvCQR publication of a study in Binh Thuan (South Coast region) was reported in 2002 [43]. The issue of imported malaria cases relative to seasonal movements of populations from free malaria to the highly malaria endemic areas for economic purposes (particularly people crossing the border with Lao and Cambodia) [34]. There are also significant overseas imported malaria cases increasing to 2.22% of total malaria case in 2015 especially from African countries where malaria is highly endemic [34, 44, 45].

Despite the success of the malaria control program, *P. vivax* infections are growing importance of in Vietnam. In order to understand the epidemiological of *P. vivax*, our study aimed to characterizing the human parasite reservoir, assessing efficacy of CQ treatment *P. vivax* as well as understanding the risk and characterizing of *P. vivax* recurrences.

2. Objectives

2.1 General objective

To improve malaria control strategies in Central Vietnam by characterizing *P. vivax* epidemiology and identifying potential targets for intervention.

2.2 Specific objectives

- To characterize *P. vivax* transmission dynamics and identify vulnerable groups among the local population;
- To assess the efficacy of chloroquine to treat *P. vivax* mono-infections within 28 days follow-up;
- To define the *P. vivax* morbidity after radical cure with chloroquine and primaquine.

3. Thesis outline

The thesis is organised in six chapters.

Chapter 1: General introduction presents the topic and rational for conducting the presented research. The objectives are outlined as well as the content of the different chapters the thesis.

Chapter 2: Literature review presents the current knowledge on *P. vivax* epidemiology and control worldwide, as well as in the Mekong Sub-region, with a focus on the current situation in Vietnam. Current challenges for the control and elimination of *P. vivax* in Vietnam are reviewed in details.

Chapter 3: Epidemiology of forest malaria in Central Vietnam: the hidden parasite reservoir. This chapter represents the first published manuscript of the thesis and reports on the burden of asymptomatic, and sub-microscopic malaria infections among some ethnic minorities (*ie*, M'nong

and Ca'Dong) living in a remote forested area of Central Vietnam (*ie*, Nam Tra My district in Quang Nam province). A risk factor analysis for malaria infection was carried out using survey multivariate logistic regression as well as the classification and regression tree method.

Chapter 4: Confirmed *P. vivax* resistance to Chloroquine in Central Vietnam. This chapter constitutes the first publication confirming *P. vivax* resistance to chloroquine in Vietnam. Indeed, *P. vivax* infected parasites still surviving while 100 mg of CQ threshold concentration remaining in blood of patients after 28-day follow-up.

Chapter 5: *P. vivax* **morbidity after radical cure: a two-year cohort study in Central Vietnam.** This chapter identifies characters of *P. vivax* recurrences including high number of recurrences after radical cure treatment under direct observation of study team, the short interval time of consecutive recurrences and the finding of prior *P. falciparum* infection as main risk factor for recurrence.

Chapter 6: General discussion. The main findings of the thesis research are discussed in the light of other publications from Vietnam and the sub-region, and implications for further elimination efforts on vivax malaria in Vietnam are analysed. Finally, research findings were used to provide recommendations for the Malaria Control and Elimination Program and suggest future perspectives for research and development to accelerate the malaria elimination agenda in Vietnam.

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CHAPTER II

Literature review on *Plasmodium vivax* epidemiology and control

Of the five Plasmodium species that affect humans, only *P. vivax* and the two species of *P. ovale* form hypnozoites, which are dormant parasite stages in the liver that cause relapses weeks to years after the primary infection is cured [1]. Hypnozoite stages are undetectable by current diagnosis tools.



1. P. vivax life cycle

Figure 1. *P. vivax* life cycle (Source: Mueller et al, 2009 [2])

In humans, the malaria parasite life cycle involves two hosts, *ie*, the human host, which harbors the asexual parasite cycle, and the vector, *ie*, female

Anopheles mosquitoes, in which the sexual cycle of the parasite takes place resulting in the production of sporozoites (the infective stage) subsequently stored in the mosquito's salivary glands. During a blood meal, sporozoites (about 10/blood meal) are inoculated into the human; they reach the bloodstream and within less than one hour reach the liver where they invade hepatocytes (Figure 1) [3]. Within infected hepatocytes, sporozoites grow in size and multiply producing thousands of merozoites, that rupture the liver cell wall releasing merozoites into the blood stream. This first cycle of asexual multiplication, which is known as pre-erythrocytic schizogony, is completed within 8-10 days.

In *P. vivax* and *P. ovale*, some sporozoites invade hepatocytes and transform into the dormant stage, i.e. "*hypnozoites*", which can persist in the liver for weeks, months or even years and cause relapses following the reactivation of their development into liver schizonts, and cause a new blood infection [2, 4].

Once in the bloodstream, *P. vivax* merozoites invade reticulocytes (immature red blood cells), which represents a small proportion (1-2%) of all red blood cells. The preference for invading reticulocytes leads to low parasite densities [5, 6]. After invading reticulocytes, the merozoites differentiate into trophozoites, and start their second cycle of mitotic divisions (erythrocytic schizogony), producing schizonts stages, which will burst realizing new merozoites into the blood circulation. Newly released merozoites will then invade new reticulocytes and continue the erythrocytic cycle, therefore being responsible for the exponential amplification of the parasitemia. This cycle of erythrocytic growth and multiplication takes 48 hours in *P. vivax* parasites and is responsible of the clinical manifestations [2, 4]. Fever usually lasts 4-

8 hours and occurs with 48-56 hour intervals [7]. The period between the parasite inoculation and the first appearance of symptoms is called incubation period, being about 14 days for *P. vivax* and *P. ovale*.

A small proportion of *P. vivax* merozoites can differentiate into gametocyte stages, which appear early in the course of infection, often before the symptoms arise [6, 7]. The estimated time of appearing *P. viax* gametocytes is about 8 days after mosquito inoculation, before they can be seen by light microscopy (30–60 gametocytes per microliter), as mosquitoes can become infected at this time. *P. vivax* gametocytes are short-lived (3–4 days) in the human circulation [8]. When female *Anopheles* mosquito bites an infected person, they suck the gametocytes and other stages of erythrocytic cycle along with blood. They reach the stomach where all the stages along with red blood cells are digested except gametocytes [9].

Within the vector's midgut, male (microgametocytes) and female (macrogametocytes) begin the sexual multiplication cycle (sporogony). While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes, about 9 to 10 days after the blood meal. The zygotes develop into ookinetes, which cross the midgut wall of the mosquito where they develop into oocysts. The oocysts grow, rupture, and release sporozoites, which make their way to the mosquito's salivary glands. During the next blood meal, the inoculation of the sporozoites into a new human host perpetuates the malaria life cycle [10]. The time required for development in the mosquito ranges from 8-21 days.

In summary, the preference for invading reticulocytes leads to low parasite densities that are more difficult to detect than *P. falciparum*, which invades blood cells of any age. A dormant liver stage (hypnozoite) can relapse weeks or months after a primary infection. The hypnozoite presents particular challenges for control and elimination of *P. vivax* malaria because it is undetectable using currently available diagnostic methods. It is not susceptible to drugs that target blood-stage forms of the parasite, and the only available drug, primaquine (PQ), causes the destruction of red blood cells (haemolysis) in people with a deficiency of the enzyme glucose-6-phosphate dehydrogenase (G6PD).

2. Epidemiology of *P. vivax* malaria

2.1 Global burden of P. vivax malaria

P. vivax is one of the five *Plasmodium* species that can cause malaria in humans and together with *P. falciparum* they represent the most important species in terms of morbidity and mortality worldwide. In 2015, WHO estimated that a total of 212 million malaria cases occurred worldwide (95% CI: 148–304 millions), most of them in the African Region (90%) with a majority of *P. falciparum* cases. *P. vivax* represented 4% (8.5 million cases estimated) of all malaria cases [11]. The *P. vivax* cases occurred in almost regions in the world as South-East Asia (58%), Eastern Mediterranean (16%) African Region (12%), America (10%) and Western Pacific regions (4%) (Figure 2). Four countries accounted for 78% of *P. vivax* cases, i.e. Ethiopia, India, Indonesia and Pakistan [11][12]. Recent reports from different endemic regions in Papua New Guinea, Brazil and India showed that not only

P. falciparum but also *P. vivax* can result in severe illness and death [13-18]. Indeed, severe manifestations of *P. vivax* malaria are similar to those caused by *P. falciparum* and include cerebral malaria, acute respiratory distress, lung injury, renal failure, hepatic dysfunction, shock and death [16, 18]. In 2015, *P. vivax* mortality estimates ranged between 4% and 39% of all malariarelated deaths outside sub-Saharan Africa [11]. However, deaths from *P. vivax* could be substantially higher as not systematically recognized, and *P. vivax* malaria should therefore not be considered as benign [19, 20]. *P. vivax* infection may indirect contribution to death in those with comorbidities, such as malnutrition, HIV and possible bacterial sepsis. Future research should include postmortem studies and detailed clinical and microbiological investigations of severe cases of *P. vivax* malaria to characterise pathogenic mechanisms and elucidate better therapeutic strategies by which fatal outcomes may be averted [95].



Figure 2. Estimated number of *P. vivax* **cases by country, 2013** (Source: W₄HO, 2015) [21]

Following the WHO Global Technical Strategy for malaria 2016-2030, targets for 2030 include at least 90% reduction in malaria incidence and mortality as compared to 2015, and malaria elimination in at least 35 countries among those endemic in 2015 [22]. These targets are challenged by remaining *P. vivax* reservoir. Research on *P. vivax* epidemiology and transmission has long been neglected and considered as benign compared to *P. falciparum*, but in the past ten years, with malaria elimination came back on the global health agenda, interest has been growing [23]. Beside its specific biological characteristics that make *P. vivax* more difficult to control than *P. falciparum* (see section § 2.3 of this chapter), increasing reports are published showing evidence of *P. vivax* resistance to chloroquine (CQ) [24-34] in all endemic areas, as well as suspected resistance to primaquine (PQ; [35, 36]) the only available treatment to prevent relapse.



2.2 P. vivax in the Greater Mekong Sub-region (GMS)

Figure 3. Malaria incidence in the GMS, 2010 (Source: Cui L, 2012) [37]

The GMS comprises Cambodia, China (Yunnan province), Lao PDR, Myanmar, Thailand, and Vietnam, and represents the most important malaria foci within South East Asia. In 2015, the population at risk of malaria was estimated at more than 152 million in the GMS [38] with the highest morbidity and mortality reported in Myanmar and Cambodia (Figure 3) [37]. From 2010 to 2015, the annual number of malaria deaths reduced by more than 90%, i.e. from 1,083 to only 85 (mortality rate from 0.069 to 0.005/100.000 pop), while morbidity reduced by over 70% between 2012 and 2015, *i.e.* from 612,843 to 165,168 confirmed cases (incidence rate from 0.38 to 0.10/1000 pop) (Figure 4A). Myanmar and Cambodia experienced the strongest reductions in malaria deaths (from 788 to 87 and from 151 to 10, respectively), and China reported no malaria death during the three continuous years from 2013 to 2015.

In 2015, the GMS reported a total of 6,9703 *P. vivax* confirmed cases , representing 42.20% of the total 165,168 malaria cases with the lowest number of cases reported in China (26 cases) and the highest (26,313cases) in Myanmar (Figure 4B) [11, 38].

Most GMS countries use the combination treatment CQ+PQ to treat *P. vivax* infected patients, except Cambodia that uses Dihidroartemisinin Piperaquin (DHA-PPQ)+PQ combination. The duration and dosing of the PQ treatment is usually 14 days of PQ at 0.25mg/kg/day (following WHO recommendations), except in China where PQ is given for 8 days at 0.75mg/kg/day [11, 38].



Figure 4. Malaria in GMS region in five years period (2010-2015). A) Changes in confirmed malaria cases and deaths in GMS from 2010 to 2015; B) Number of confirmed *P. vivax* cases by country in GMS, 2015 (Data source WHO, 2016 [11])









Figure 5. Global *P. falciparum* and *P. vivax* endemicity in 2010. A) The spatial distribution of *P. falciparum* malaria endemicity 2010 World; B) The spatial distribution of *P. vivax* malaria endemicity 2010 World [39]

2.3 Challenges for *P. vivax* control and elimination

We listed below several biological and epidemiological characteristics of *P*. *vivax,* which challenges its control.

It can survive in cooler climates. (Figure 5)

P. vivax can develop and survive in the relatively cooler climates of temperate countries [20]. *P. vivax* sporozoites develop faster than *P. falciparum* inside infected female mosquito at equivalent temperatures, which contributes to its exploitation of a wider geographic range [40], and is therefore, less susceptible to interventions aiming at reducing mosquitoes longevity [20]. *P. vivax* can undergo sporogony in a wide range of anopheline species at subtropical temperatures (16 °C for *P. vivax* versus 18 °C for *P. falciparum*), while *P. vivax* sporogony in the vector is shorter (~10 days at 25 °C) than for *P. falciparum* (12 days) [20].

It is less responsive to conventional methods of vector control.

In many areas where *P. vivax* malaria is common species (such as in SEA and South America), mosquitoes bite early in the evening (before individuals are protected by bed nets), obtain blood meals outdoors and resting outdoors [20]. Therefore, conventional vector control tools such as Insectiside treated nets (ITNs) and Indoor residual spraying (IRS).

It is more difficult to detect using current diagnostic techniques.

P. vivax blood-stage infections tend to be of lower density than *P. falciparum* infections resulting in often missed diagnosis by standard microscopy and RDT and consequently the underestimation of the true prevalence of *P. vivax* infections. This is especially true in low transmission and pre-elimination

settings, where submicroscopic infections represent large proportions of the human malaria parasite reservoir. Some studies indicated that very lowdensity *P. vivax* infections do contribute to ongoing transmission [42, 43]. In addition, *P. vivax* dormant liver stage (hypnozoites) cannot be detected by currently available diagnostic tools. Hence, there may be a large reservoir of infected people with *P. vivax* but are unaware of their condition. In addition, *P. vivax* gametocytes appear in the blood of infected patients before the development of symptoms therefore, transmission may occur before an infection is diagnosed or treated [20].

A single infection can give rise to multiple episodes of malaria.

P. vivax infected patients carry dormant liver forms (hypnozoites) which can reactivate months or years after the primary infection, and increases the transmission potential of this species. Unfortunately, this parasite stage cannot be detected with currently available diagnostic tools which represents a challenge for control and elimination programs as it will not be detected in malariometric surveys or active case detection campaigns.

P. vivax infections are often characterized by the presence of two or more genetically distinct parasites in the same individual [44-46]. Multiclonal infections can arise from a single mosquito bite carrying a mixture of parasite clones or from inoculation by different mosquitoes carrying single clones. The number of parasite clones in a patient varies greatly, and some infections contain up to nine clones [44]. In addition, even without mosquito bite, the dormant liver stage can awaken and trigger multiple vivax malaria episodes contributing to increased clonal diversity.

Following WHO malaria terminology, recrudescence and relapse are defined as following: recrudescence is recurrence of asexual parasitaemia of the same genotype(s) that caused the original illness, due to incomplete clearance of asexual parasites after antimalarial treatment. While relapse is recurrence of asexual parasitaemia in *P. vivax* infections arising from hypnozoites.

Tropical *P. vivax* relapses at three week intervals if rapidly eliminated antimalarials are given for treatment, whereas in temperate regions and parts of the sub-tropics *P. vivax* infections are characterized either by a long incubation and relapse - in both cases approximating 8-10 months. The number of sporozoites inoculated by the anopheline mosquito is an important determinant of both the timing and the number of relapses. The intervals between relapses display a remarkable periodicity which has not been explained [96].

Treatment of liver-stage parasites requires a 14-day course of primaquine.

So far, primaquine is the only drug available on the market which is effective against hypnozoites. The radical treatment of this parasite stage requires patients to take primaquine every day for 14 days [47]. In vivax endemic areas, many patients have difficulty complying with such a lengthy treatment regimen, hence poor adherence to treatment contributes to the poor of effectiveness of primaquine [48]. Tafenoquine, another 8-aminoquinoleine, is under investigation [49] as a single-dose radical cure for *P.vivax* malaria but it also causes hemolysis in patients with deficiency in glucose-6-phosphate dehydrogenase (G6PD)[47].

G6PD deficiency

Primaquine treatment can produce serious side-effects, because it is associated with potentially fatal haemolysis in individuals with inherited deficiency of G6PD enzyme [47]. In addition, this drug cannot be used in pregnant women and infants, which represent the groups most vulnerable to vivax malaria. Recently, safe primaquine therapy is guided by testing for G6PD status before prescription [20, 50] following the most recent WHO treatment guidelines [51]. However, current tests for G6PD deficiency are not easy to use and relatively expensive; thus, many clinicians are reluctant to prescribe primaquine to patients whose G6PD status is unknown [52].

3. P. vivax malaria in Vietnam

3.1 Brief history of malaria control in Vietnam (eradicationelimination-control)

In 1953, 23% of the Vietnamese population was living in malaria endemic areas [53]. In accordance with the WHO's Global Malaria Eradication Program, a malaria program was started in northern Vietnam in 1958 focusing in case management and IRS using Dichlorodiphenyltrichloroethane (DDT) [54]. Despite the Vietnam War (1955–1975) malaria morbidity and mortality reduced for most of the 1970s. The program was expanded in 1976 after North and South Vietnam reunited and over 600,000 malaria cases and 1,500 malaria-related deaths were reported this year.

In 1986, over a million cases were recorded with almost 2,000 deaths. Malaria continued to increase with a peak in 1991, when more than 4,000 deaths ocurred [55]. In response to this situation, the National Malaria Control

Program (NMCP) was established in 1992. Used strategies included early detection and prompt treatment of all malaria cases, introduction of artemisinin derived drugs (used as monotherapies), distribution of ITN, selective indoor spraying (in areas with low ITN coverage) and health education at community level (on the importance of malaria and ITN use) supported by media campaigns. From 1991 to 2010, the number of malaria deaths decreased by more than 99 percent, *i.e* from 4,646 to 21, while the number of reported malaria cases reduced by 95%, *i.e.* from 1,091,251 to 54,297 [55, 56]. ITN provides an effective community protection only when at least 80% of the population uses bed-nets regularly. The population protected went from 4 million in 1991 to 12 million in 1997/98. The population using ITN (for personal protection) increased from 300,000 in 1991 to 10 million in 1997. Between 1992 and 1997, the new drug artemisinin was safely and successfully introduced and severe malaria cases were reduced from 21,000 in 1992 to 1,500 in 1997. It is the drug of choice for all confirmed cases of *P. falciparum* and for any severe case even before confirmation. [57].

3.2. The National Malaria Control and Elimination Program

At the end of 2010, the Vietnamese Government and the Ministry of Health (MoH) highly supported NIMPE started the Malaria Control and Elimination Program (NMCEP) to achieve the goal to eliminate malaria by 2030 [58]. However, as in other countries in the Asia Pacific region, Vietnam faces numerous challenges to malaria elimination, including the fast spread of drug-resistant malaria parasites and the continuous populations movement between malaria-free and malaria-endemic areas. Moreover, the residual foci of malaria transmission are situated in the hardest to reach areas and affects the most vulnerable populations, *i.e.* remote, forested areas along international

borders and in the Central highlands inhabited by impoverished poorly educated ethnic minorities. This requires a highly responsive and comprehensive surveillance system to effectively track down and treat all malaria cases and infections.

After the first five years (2011-2015) of the new NMCPE, malaria morbidity and mortality was reduced by 44% (from 16,612 in 2011 to 9,331 in 2015) and 79% (from 14 in 2011 to 3 in 2015) respectively [59, 60]. A second 5year 2016-2020 phase is now ongoing focusing on elimination malaria in low endemic areas and with the target to eliminate malaria from 40 provinces. In 2017, a total of 4,548 confirmed malaria infections and 6 deaths were reported by the NMCEP [61].

3.3. Evolution of *P. vivax* morbidity-mortality since 1991

Figure 6A shows the evolution of malaria mortality and species specific morbidity since 1991. *P. falciparum* and *P. vivax* are both prevalent malaria species in Vietnam and the relative importance of *P. vivax* increased from 18% (1991) to 42% (2016) (Figure 6B).

In 2016, a total of 4,161 confirmed malaria cases were reported nationally (incidence = 0.05 cases/1000 pop) with only 3 confirmed malaria deaths (all due *P. falciparum*) and no malaria out-break. *P. vivax* represented 42.1% (n=1,750) of all malaria cases and concentrated in 10 provinces (83.54% (1,462/1,750)) mainly in the central and southeast part of the country (Figure 7). The highest incidence of *P. vivax* cases were observed in Binh Phuoc (n=354), Gia Lai (n=289), Dak Lak (n=139) and Quang Nam provinces (n=135) [62] representing 52% of all vivax reported cases.


Figure 6. A) Evolution of malaria morbidity and mortality from 1991 to 2016. B) Evolution of human malaria parasite species distribution (%) between from 1991 to 2016 (Source: Annual malaria report of NIMPE, 1991-2016)

For economic purposes, people move between malaria-free and –endemic provinces as well as across international borders, which makes it difficult for the health system alone to monitor these populations. These populations can spread malaria parasites between endemic and non-endemic areas which raises serious concerns for the success of current malaria elimination efforts [62]. The threat is even more serious when considering that populations living in malaria endemic areas, *ie* local ethnic minorities and migrants with forested-related occupations, often carry asymptomatic and sub-microscopic malaria infections, which represent as many sources of continued transmission [63-65] and possibly reintroduction of malaria in recently freed areas from malaria where vectors are still present such as most provinces in the north and south of Vietnam.

Currently, border malaria is challenging malaria control and elimination effort in Vietnam. In 2017, Vietnam NMCP recoded 207 imported malaria cases from Cambodia and 148 from Laos [94]. Almost imported malaria cases are detected among forest goer group who usually go to work in the forested area in Laos and Cambodia where they infected malaria. To date, artemisinin resistance has been detected in 5 countries of the GMS: Cambodia, Lao People's Democratic Republic (PDR), Myanmar, Thailand and Viet Nam. To reach the goal: elimination of all species of human malaria across the GMS by 2030, the collaboration between GMS countries focused on 1) improving access to high quality drugs; 2) ensuring drug efficacy and detecting drug resistance; 3) strengthening surveillance of malaria cases; 4) reaching migrant & mobile populations [97].



Figure 7. Distribution of confirmed *P. vivax* cases by province, 2016 and four provinces had highest number of *P.vivax* cases (Source: NIMPE, 2016) [62]

3.3 Current malaria epidemiological stratification map and malaria control & elimination strategies

Since 2003, a malaria epidemiological stratification has been implemented in all 63 provinces/cities of Vietnam [66]. This tool aims at identifying the different levels of malaria endemicity and risk of infection per province in order to develop more effective interventions by setting priorities and targeting specific malaria control and elimination measures depending on transmission intensity of each province. This tool also allows to identify areas where the malaria program needs extra efforts and helps to make the best use of limited resources [66].

Five different zones have been defined according to the number of indigenous malaria cases (*i.e.*, locally acquired malaria cases) as following: *i*) malaria free areas; *ii*) zones at risk of malaria resurgence; *iii*) low endemic areas; *iv*) moderate-, and v) high endemic areas (Table 1). The main indicators for classification are the average number of confirmed cases per 1,000 population over the 5-year period, and the presence of at least one indicator such as: presence of malaria vectors, socio-economicly disadvantaged or border communes, poor health system, drug resistant parasites, chemically resistant mosquitoes, and migratory populations. Indeed, the indicators were scored as following: i) Annual incidence rate (ARI) <1 case/1,000 pop/year=5 points; ARI: 1-<5 cases/1,000pop/year=10 points; 5 ii) iii) ARI >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; and the sum of the scores will be used to define the level of endemicity and priority for interventions (Table 1). Following the last stratification in 2014, the malaria-free areas included 5,840 communes (52.37% from total) with 64,541,280 people (64.64% of total population). The areas at risk of malaria resurgence included 3,448 communes (30.92%) with 21,445,395 inhabitants (22.89%). The low endemic areas included 1,095 communes (9.82%) with a population of 7,710,946 (8.23%); the moderately endemic areas consisted of 529 communes (4.74%) with a population of 2,813,221 (3.00%); and the high malaria endemic area consisted of 240 communes (2.15%) with population of 1,167,628 (1.25%).

The results of the three successive malaria stratifications in 2003, 2009 and 2014 are shown in Figure 8. This shows a steady reduction in the number of highly endemic communes and an increase of the low endemic communes, while malaria free areas and those at risk of resurgence, remained stable. The remaining areas at moderate and high risk concentrate along international borders (Laos and Cambodia) and in Central and Southeast Vietnam.

Table 1. Stratification of malaria endemicity in 2014 with main control strategies [66]

Areas-Characteristics	Intervention focus
ZONE 1 Malaria free areas (no local transmission) • Prevention of malaria re-introduction	• Early diagnosis & prompt treatment (ED&PT) of imported malaria cases.
ZONE 2 Risk of malaria resurgence • Prevention of malaria re-introduction	 ED&PT of imported malaria cases. Standby treatment (=self-treatment in people going to malaria endemic areas); Strict management and investigation of imported cases, regular surveillance (PCD&ACD); identification and monitoring of migrants; Provision of ITNs for people going to malaria endemic areas.
ZONE 3 Low endemic area • Elimination phase • Confirmed malaria cases= >0-1/1000pop	 ED&PT of imported malaria cases; standby treatment; ACD of malaria, investigation and mapping 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.

ZONE 4 Moderately endemic area • Pre-elimination phase • 1-5cases/1000pop	 cases, PT+ primaquine (PQ) for <i>P</i>,<i>f</i> gametocytes and anti-relapse of <i>P</i>.<i>v</i> and <i>P</i>.<i>o</i>; Regular malaria surveillance; monitoring 39ntimalarial drug resistance; GIS for malaria infections and vectors, consolidate peripheral health services, involve private sector in diagnosis, treatment, control and the selling of 39ntimalarial drugs; Annual LLINs or ITNs distribution for local people, IRS where<80% people sleep under nets and in epidemic prone areas; Collaboration with other ministries and sectors, strengthening collaboration with military in malaria control.
ZONE 5 High endemic area • Control and consolidation phase • >5cases/1000pop	 Mobilization to sleep under ITNS especially in the fields, or forest; ED by LM or RDTs for 100% suspected cases, PT +PQ 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 directly observed (DOT); Monitoring malaria drug resistance; GIS mapping 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. ITNs or LLINs annually for local people; prioritize IRS and ITNs in epidemic prone areas; IRS where <80% of people sleep under nets. In high risk areas, IRS and ITNs 2 times/year if ITN coverage<80%. Multiple collaborations with other ministries, and military; promote community-based malaria prevention.

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; ED=Early detection; PT=prompt treatment; LM=Light microscopy; RDTs=Rapid diagnosis test; IRS=Indoor residual spraying



Figure 8. Malaria epidemiology stratification in Vietnam in 2003, 2009 and 2014 (Source: NIMPE, 2014) [66]

Currently, the NMCEP is aiming 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 [67]. Following the stratification map of 2014, the strategy for preventing malaria re-introduction is applied in zone 1 including 5,840 communes (52.37%), malaria elimination in zone 2 with 53,448 communes (30.92%) and malaria pre-elimination in zone 3 with 1,095 communes (9.82%). However, the control phase is done in parallel to the elimination phase and covers Zone 4 & 5, which consists of 529 communes (4.74%) and 240 communes (2.15%) respectively [66].

Based on results of 2014 malaria stratification (Table 1) the main recommended malaria interventions are as follows:

- Early diagnosis and prompt treatment (EDPT) are common rules of PCD in all health centers (especially in village by VHWs) and regular ACD campaigns are carried out by mobile teams of district health center (DHC) or provincial malaria center (PMS). 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 [68]
- Vector control activities, including IRS and ITNs/ Long lasting insecticidal nets (LLINs) distribution, are carried out annually in the remote and endemic areas before and during the malaria season. LLINs 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 [69].

- Surveillance activities, in both control and elimination phases, are updated with new malaria information system providing real-time malaria case reports, both daily and weekly via the internet. Confirmed malaria cases are directly reported using "malaria case report form" by all commune health centers to their respective DHC and PMC, as well as the regional institutes (IMPEs in Quy Nhon and Ho Chi Minh) which will decide to carry out case and foci investigation according to case stratification key. This allows the NMCEP to assess the malaria situation in real time and to respond in a timely manner with adequate interventions [69].
- Health education activities on malaria prevention are implemented in all communes. The different IEC materials including agendas, leaflets, flip flops are distributed to the communities at risk of malaria during IRS campaigns, LLINs distributions, ACD campaigns by health staffs and VHWs. The malaria prevention message also sends to local people via radio, TV in ethnic minority language. In addition, the World Malaria Day is organized annually in malaria endemic areas to raise community awareness [70].
- Research activities focus on several challenges such as knowledge gaps on *P.vivax* epidemiology, the contribution of asymptomatic and submicroscopic infections to malaria transmission, antimalarial drugs resistance, population movements between endemic and malaria-free areas, and imported malaria parasites from other countries with high malaria prevalence [66, 71].
- Planning and evaluating activities of the NMCEP remain difficult due to the high proportion of asymptomatic and sub-patent microscopic

infections, which lead to substantial underestimations of the actual human malaria parasites reservoir [65]. This is due to the limited sensitivity of current detection methods by LM and RDT. Therefore, new and highly sensitive diagnostic tools (based on molecular and serological techniques) are necessary accurately characterize the current reservoir of infections as well as monitor and evaluate the results of the current Malaria Control and Elimination efforts in order to achieve the goal of a malaria-free Vietnam in 2030.

3.4 P. vivax treatment and drug resistance

Malaria diagnosis is based on clinical signs and the identification of malaria parasites in patients' blood. Early clinical signs are unspecific as they are common to other diseases. Current WHO treatment guidelines recommend prompt parasitological confirmation by microscopy or RDT before antimalarial treatment. The golden standard for laboratory diagnosis is the light microscopy examination of Giemsa-stained thick and thin blood films [72]. However, the limitation of microscopy and RDT is that it may not detect patients with very low malaria parasite density. Indeed, RDT can detect parasite density from 100 to 200 parasites/µl and LM from 60 to 100 parasites/µl. Currently, PCR-based techniques have been extensively used for diagnosis and quality control due to their high sensibility (<1 parasites/µl of blood) and specificity, particularly with low parasitemia and mixed infections [72-74]. Nested PCR and real-time PCR methods are widely used for malaria diagnostic and research, though these techniques are not easily deployable in the field. Recently, loop-mediated isothermal amplification (LAMP) technique has been recently developed to detect malaria parasites in field conditions. Its sensitivity is similar to that of standard PCR, while results are available within 2 hours [75-77].

Chloroquine (CQ) has been the first-line therapy of choice for blood stage *P*. *vivax* parasites since 1946 [4, 78, 79]. This drug clears *P*. *vivax* parasites within 72 hours of the first dose. The long elimination half-life (20 to 60 days) provides therapeutic blood levels able to eliminate early relapsing liver hypnozoites from the blood [4, 42, 80].

Primaquine (PQ) was first synthesized in the United States of America between 1941 and 1945. Currently it is still the only drug available with a schizonticidal activity against hypnozoites (tissue schizontes) (*ie*, eliminate dormant liver forms). PQ is active against parasite asexual stages in the liver, as well as asexual trophozoites and gametocytes in the blood. However, the mechanisms of action of PQ against hypnozoites are still unknown. Based on clinical trials conducted in the 1950s, PQ is effective in clearing hypnozoites and preventing relapses using a regimen of 15 mg/kg for 14 days [81, 82].

In Vietnam, the recommended PQ radical cure regimen for *P. vivax* has been modified several times over the past twenty years both in length and dosage as shown below:

- 1997: 0.5mg/kg/day PQ for 5 days [83];
- 2003: 0.25mg/kg/day for 10 days [84];
- 2007: 0.5mg/kg/day for 10 days [85];
- 2009 : 0.25mg/kg/day for 14 days [86].

These different regimens were always recommended together with a 3-day course of CQ (25mg/kg in total). Thus, since 2009, the first line treatment for uncomplicated *P. vivax* malaria infections is a 14-day PQ regimen at half dose compared to the 2007 guidelines. [4]. The reasons for this change are based on issues related to the duration, dosage and treatment adherence [87-89]. In addition, the main adverse event of PQ consisting of acute hemolysis in people with a genetic deficiency of glucose-6-phosphate dehydrogenase (G6PD), also contributed to the change in treatment policy [81]. In 2007, following WHO treatment guideline for Chesson *P. vivax* type in Southeast Asia countries, NMCP used PQ 0.5mg/kg/day in 10 days for reducing inadherence PQ treatment. However, the malaria annual reports within 2007-2009 recoded the fear to hemolysis of health staffs when they treated PQ without testing of G6PD deficiency and the patients hardly took the high dose of PQ regiment. Therefore, NMCP returned to 0.25mg/kg/day for 14 days in 2010 (Dr. Nguyen Van Hong personal communication).

The first reports of CQ resistant *P. vivax* (PvCQR) were documented in 1989 when Australians repatriated from Papua New Guinea (PNG) failed routine treatment [87]. Studies in Indonesia and PNG have shown treatment failure risk up to nearly 100% [4, 6, 26, 90]. Currently, reports of recent emergence of PvCQR strains have been documented in almost all vivax endemic countries [24-34]. In Vietnam, information on *P. vivax* resistance to CQ is very scarce with only one report in 2002 from the Central Coast region (Binh Thuan province) of reappearance of *P. vivax* parasite within 28-day after CQ treatment in 2002 [91]. However, *P. vivax* sensitivity to CQ was regularly monitored between 2006 and 2011 by the NIMPE (total 350 patients) and 100% ACPR was reported almost continuously within the six sentinel sites in

Central Vietnam [92]. Later in 2011, the study in Quang Nam province (see Chapter 4) confirmed *P.vivax* resistance with CQ blood concentrations higher than the minimal inhibitory concentrations (MIC) within 28 days after treatment by CQ+PQ [85]. Retrospective data from 2002 to 2011 on CQ efficacy for the treatment of uncomplicated *P. vivax* malaria in Vietnam is summarized in Figure 9. The overall 13 studies were carried out in 7 provinces in Central and South part of Vietnam. The *P. vivax* patients ranks from 14 (Dak Nong, 2007) to 113 (Binh Thuan, 2002) and the adequate clinical and parasitological response (ACRP) was recorded 84% as lowest in Binh Thuan province.

PQ resistance is unknown because assessments of efficacy of PQ have been confounded by variations in relapse patterns and study design such as duration of follow-up. Efficacy of PQ treatment seems to be more dependent on the total PQ dose than on the duration of treatment. In 2007, a study evaluating safety and efficacy of a short artesunate– primaquine combination treatment against vivax in adult patients from Viet Nam showed that 22.5 mg PQ daily for 7 days given 0, 12, 24 and 36 h after 200 mg artesunate was effective during a follow-up of 28 days. Only one of 28 patients (3.6%) had a recurrence on day 28 [93]. Very few publications on adherence to PQ are available from Vietnam, but one study done in Thailand reported on this topic suggesting why the effectiveness of PQ is often poor [48].



Figure 9. CQ efficacy for the treatment of uncomplicated *P. vivax* malaria in central Vietnam, data from 2002 to 2011 (Source: NIMPE, Hanoi). %ACPR (Number of patient's studies)

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CHAPTER III

Epidemiology of forest malaria in Central Vietnam: the hidden parasite reservoir

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Abstract Background

After successfully reducing the malaria burden to pre-elimination levels over the past two decades, the national malaria programme in Vietnam has recently switched from control to elimination. However, in forested areas of Central Vietnam malaria elimination is likely to be jeopardized by the high occurrence of asymptomatic and submicroscopic infections as shown by previous reports. This paper presents the results of a malaria survey carried out in a remote forested area of Central Vietnam where we evaluated malaria prevalence and risk factors for infection.

Methods

After a full census (four study villages = 1,810 inhabitants), the study population was screened for malaria infections by standard microscopy and, if needed, treated according to national guidelines. An additional blood sample on filter paper was also taken in a random sample of the population for later polymerase chain reaction (PCR) and more accurate estimation of the actual burden of malaria infections. The risk factor analysis for malaria infections was done using survey multivariate logistic regression as well as the classification and regression tree method (CART).

Results

A total of 1,450 individuals were screened. Malaria prevalence by microscopy was 7.8% (ranging from 3.9 to 10.9% across villages) mostly *Plasmodium falciparum* (81.4%) or *Plasmodium vivax* (17.7%) mono-infections; a large majority (69.9%) was asymptomatic. By PCR, the prevalence was estimated at 22.6% (ranging from 16.4 to 42.5%) with a higher proportion of *P. vivax* mono-infections (43.2%). The proportion of sub-patent infections increased with increasing age and with decreasing prevalence across villages. The main risk factors were young age, village, house structure, and absence of bed net. **Conclusion**

This study confirmed that in Central Vietnam a substantial part of the human malaria reservoir is hidden. Additional studies are urgently needed to assess the contribution of this hidden reservoir to the maintenance of malaria transmission. Such evidence will be crucial for guiding elimination strategies. **Keywords:** Malaria, sub-patent infections, elimination, Central Vietnam

Background

The past 20 years of continued malaria control efforts have resulted in the elimination of this disease in several provinces of Northern and Southern Vietnam [1]. In 2011, the Vietnamese Government officially launched the National Malaria Control and Elimination Programme aiming at malaria elimination for the whole country by 2030 [2]. However, such an ambitious goal faces several challenges that include forest malaria, seasonal population movements (internal and across international borders) and emerging drug resistance.

Currently, most malaria morbidity (18,387 confirmed cases in 2012) and malaria deaths (eight in 2012) occur in 21 out of 58 provinces (=25% of total population) and are located in Central and Central-Southern Vietnam [3], where standard vector control interventions are unable to interrupt forest malaria transmission and where *Plasmodium falciparum* resistance to artemisinin derivatives has been reported [4]. Asymptomatic malaria infections are common in remote and forested areas of Central Vietnam [5-7], particularly among local ethnic minorities in which the burden of *Plasmodium vivax* is particularly high. A recent survey carried out in Ninh

Thuan Province (Central-Southern Vietnam), in which filter paper blood samples were analysed by molecular techniques, showed the presence of a largely hidden human reservoir of malaria infections with numerous subpatent infections (detected only by PCR but not by microscopy) including mixed infections with Plasmodium malariae and Plasmodium ovale [8]. Besides the difficulty of identification by standard microscopy, P. vivax and P. ovale may have dormant liver forms (hypnozoites) that can reactivate at varying times after the primary infection. Vietnamese treatment guidelines recommend the use of both a three-day course of chloroquine (0.25 mg/kg) and a 14-day course of primaquine (0.25 mg/kg/day) to clear both peripheral blood and liver stages of infection. However, a 14-day course of primaguine is rarely followed due to fears of haemolysis in glucose-6-phosphate dehydrogenase deficient (G6PDd) individuals [9]. In addition, compliance to the 14-day primaquine treatment is usually low. The latter issue was addressed by recommending in January 2007 (decision number 339/QĐ-BYT) a shorter but higher dosage of primaquine, i.e., ten days at a daily dose of 0.5 mg/kg. A cohort study was set up in Quang Nam Province from 2009 to 2011 to assess the short- and long-term efficacy of the new regimen. The present paper reports the baseline malaria prevalence and related risk factors among the study population before the start of the cohort.

Methods

Study site and population

The study was carried out in four villages located in two communes (Tra Leng and Tra Don) situated in Nam Tra My district in Quang Nam Province (Central Vietnam) (Figure 1). Study villages were located in a remote forested valley accessible only on foot (five hours) or motorbike (two hours) on a mountain track from the nearest health centre in Tra Don commune. Villages were extremely scattered, with households grouped in clusters of four to 45 houses situated at variable distance from each other. The number of clusters varied by village with four clusters in Village 1, two in Village 2, nine in Village 3, and five in Village 4. All study clusters were served only by the CHC in Tra Leng since the one in Tra Don commune was too far. Village 3 and 1 were located along the way to and around the CHC, respectively, while Villages 2 and 4 were situated at 4- and 3 hours walking distance (for the farthest clusters) from the CHC. In addition, there was a river between the centre of the commune and Village 4 whose access was almost impossible during the heavy rains.

The population mainly belonged to the M'nong and Ca Dong ethnic groups living in very poor socio-economic conditions, mainly subsistence farming, practising slash-and-burn agriculture in forest fields with maize, manioc and rice. Malaria transmission is perennial with two peaks, one in May-June and the other in October-November, with the two main vectors species being *Anopheles dirus sensu stricto* and *Anopheles minimus sensu stricto* [10, 11]. Malaria control activities are based on free-of-charge, early diagnosis and treatment with an artemisinin-based combination (ACT; dihydroartemisinin-piperaquine) and regular indoor residual spraying (IRS; alpha-cypermethrin) as bed net use was not very popular in the study area at the time of the survey (Nguyen Van Van, personal communication).

The Commune Health Centre (CHC) located in the centre of the commune (Village 1) was hardly accessible for Village 4 during the rainy season because of heavy rains and flooding. The local health staff (one midwife, three nurses, one microscopist, and one pharmacist) provided free-of-charge health care with the support of village health workers (VHWs).

Data collection

In February, 2009 a full census of the study population (1,810 individuals) was carried out to collect household as well as individual socio-demographic data (gender, age, location, occupation, assets, distance to the



Figure 1. Map of the study area showing malaria prevalence in the 20 study clusters (by microscopy and PCR)

fields, number of available bed nets per household, housing structure, etc.). Each resident in the study area was allocated a unique ID code. Each house was mapped using a geography position system device (Garmin eTrex Legend HCx Personal Navigator) [12].

In April 2009, the entire study population was screened for *P. vivax* infections to identify potential study participants for a cohort to be followed prospectively. This started by informing first all commune, village and household leaders on the objectives and study procedures and then the individual study subjects, who were all invited to be screened after oral informed consent. During the screening, each participant was interviewed for previous malaria symptoms during the previous 48 hours, the axillary temperature was checked and a blood slide collected for light microscopy (LM). Confirmed malaria infections were treated according to the national treatment guidelines.

In addition to blood slides taken during the screening, an additional blood sample was taken for haemoglobin measurement and for later molecular analysis (PCR) in a random sample of study participants (n=327). This was done by randomly choosing one individual in each household after blindly drawing an ID number among those allocated to the house during the census.

If the selected subject was temporarily absent, the survey team would return later; however, if the subject was absent for a long time or not willing to participate, a second drawing would be done. Survey participants (i.e., with additional blood samples) were asked to give their written informed consent (parent/guardian for children) after being explained the purpose of the additional sampling and investigations. Among these subjects, a face-to-face interview was done to collect data on the different outdoor activities in and outside the community, sleeping habits, as well as malaria prevention measures. For children under 12 years old, the parent/guardian would answer the questions.

Laboratory procedures

Thick and thin films were stained with a 3% Giemsa solution for 45 minutes. The number of asexual forms per 200 white blood cells (WBCs) was counted and parasite densities were computed assuming a mean WBCs count of $8,000/\mu$ l. Gametocytes were also counted. A slide was declared negative when no parasite was seen after counting 1,000 WBCs. All slides were read independently by two expert microscopists. In case of discrepant results, they re-examined the slide together until agreement was reached. Quality control of blood slides was done on all positives and 10% of negative blood slides by

a senior laboratory technician at the National Institute of Malariology, Parasitology and Entomology (NIMPE), Hanoi; in case of disagreement, a second senior technician would re-read the slide until an agreement was reached.

Haemoglobin concentration was measured with the HemoCue Hb 301 device following the manufacturer's instructions [13]. Filter paper blood samples (FPBS) were dried outside in direct sunlight and kept in individual, sealed, plastic bags containing silica gel. All FPBS were stored at 4°C in the CHC refrigerator before being shipped to NIMPE, Hanoi, where they were kept at -20°C. DNA extraction was done using the QIAamp DNA Micro Kit (Qiagen, Hilden Germany), and a species-specific, semi-nested, multiplex PCR (SnM-PCR) was performed to detect *P. falciparum, P. vivax, P. malariae,* and *P. ovale* [8]. The PCR products (5 µl) were subjected to electrophoresis on a 2% agarose gel *in 0.5X TAE buffer* for 90 minutes at 100V. 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. Cross-contamination during handling was checked for by implementing negative controls in each step from extraction to the nested PCR step. Quality control was done on 10% of the samples for which the SnM-PCR was repeated blindly by a senior technician. In case of discrepancy, the sample was re-analysed until agreement was reached.

All survey samples were analysed for G6PD deficiency at the Shoklo Malaria Research Unit, by genotyping for the Viangchan mutation following a modified protocol published by Nuchprayoon *et al.* [14]. DNA was extracted using the Saponin-Chelex method [15]. Genotyping for the Viangchan mutation (871G>A) was performed by PCR/RFLP method using published primers [14] and MyTAqTM DNA polymerase (Bioline, UK) with the following amplification conditions: initial denaturating step at 95°C (5 min) followed by 30 cycles of 95°C (30 sec), 57°C (20 sec), and 72°C (15 sec) and final elongation step at 72°C for 7 min. Amplified fragments were digested with XbaI enzyme and visualized on a 3% agarose-nusieve gel. Quality control was performed on 10% of randomly selected survey samples; in case of disagreement, the sample was re-analysed by another senior technician.

Data management and statistical analysis

Sample size: According to the provincial malaria station data on surveys carried out in April-May, the overall parasite rate was around 16% (ranging

from 5 to 39% across hamlets) and the prevalence of *P. vivax* at 9%. The sample size was calculated by assuming a minimal prevalence of 9%, with 3% precision at 5% significance level and adding 10% security margin; a total of 330 individuals were needed for the survey ("CSample" command/EpiInfo6). Therefore, to simplify sampling procedures, one individual in each house visited during screening was randomly selected to be included in the survey.

Data were double-entered and cleaned using Epidata version 3.1 free software [16]. The data set was analysed using STATA version 11 (Stata Corp, College Station, TX, USA). Descriptive statistics were used to compute baseline socio-demographic characteristics as well as malariometric indices by village, and significant differences were tested for using either a Chi-square test or Student t-test as required, and a p-value <0.05 was used as cut-off for significance.

Three different variables for livestock ownership (number of i) buffaloes, ii) cows, and iii) pigs) were considered as the best proxy (after discussion with household leaders) for the economic status of the households as all inhabitants were subsistence farmers and generally poor. In order to aggregate multiple
variables to a single measure of economic status, a principal component analysis was performed [17]. Using the factor scores from the first principal component as weights, an index was created for the economic status of each household then the index were categorized by dividing the score into tertile.

The survey design (survey dataset) was taken into account using the svycommand in STATA, with villages as *strata*, and household sizes as p*weights*. A survey logistic regression (*"svy"* command in STATA) was used to carry out a multivariate adjusted analysis for the risk of malaria infection (determined by PCR). Moreover, a classification tree analysis (CART; Salford Systems Inc, CA, USA) was performed to explore the relationship and rank the relative importance of risk factors for all malaria infections identified by PCR, as well as for patent infections only (detected both by PCR and microscopy). Sub-patent (or sub-microscopic) malaria infections are detected by PCR only. The CART analysis is a non-parametric method enabling more direct and flexible analyses since, unlike logistic regression models, it allows for co-linearity and multiple interactions between different independent variables [18]. Briefly, the building of the classification tree starts with the root node, which contains the entire set of observations. CART then finds the best possible variable to split the root node into two child nodes, by identifying the best splitting variable that maximizes the average 'purity' (homogeneity) of the two child nodes. To improve the stability of the CART model, a ten-fold cross-validation method was applied, and the best tree was selected by choosing the smallest tree within one standard error of the minimum error. CART also provides a ranking power of each predictor variable.

Ethical clearance

Ethical clearance was obtained from both the ethical committee of NIMPE in Hanoi and the University of Antwerp. The fundamental principles of ethics in research on human participants were upheld throughout the project. All study participants gave their informed consent after being explained the study procedures as well as their right to withdraw without prejudice for themselves or their families.

Results

A total of 1,810 individuals were identified during the census, representing 352 households distributed in 20 clusters within the four study villages (Table 1). Clusters varied substantially in size (range: four to 45 households) and

distance from each other (Figure 1). The M'nong ethnic group, mainly living in Villages 1-3, was the most represented (79.9%), while all the Ca Dong (19.3%) lived in Village 4. The study population was young (median age=16 years), poorly educated and with low socio-economic status. Most of the houses were provided by the government, with metal roofs (93.7%) and wooden walls (88.1%). Bed net coverage (untreated nets) was very low with more than 75% of households having no

nets and only 7.7% had a sufficient number to achieve a coverage of maximum two persons/net. Most adults, both males and females, were farmers (90.5%), practising slash-and-burn agriculture in forest fields, as well as cinnamon tree cultivation; the wealthiest families had some livestock.

A total of 1,450 individuals (80.1%) were screened for malaria by LM (Table 2) and their socio-demographic characteristics were similar to those of the whole population. The main reason for non-participation was absence at the time of the screening because of schooling (pupils, students) or to

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Table 1. Dasenne characteristics of the study population								
Study population N = 1,810	n	%						
Village 1	407	22.5						
Village 2	305	16.8						
Village 3	751	41.5						
Village 4	347	19.2						
Gender								
Male	929	51.3						
Female	881	48.7						
Age groups								
<9	568	31.4						
10 19	443	24.5						
20 29	321	17.7						
> 30	478	26.4						
Median age: [range]	16. [1. 88]							
Ethnic groups	10, [1, 00]							
Cadong	350	193						
M'nong	1 447	79.9						
Kinh and others	13	0.7						
Education level (age >18 years $n=834$)	15	0.7						
None	230	27.6						
Flementary	230	27.0 48.2						
Elementary Secondary and above	402	40.2						
Secondary and above $(aga > 18 yaara = 224)$	202	24.2						
Occupation (age >18 years, n=834)	27	2.2						
Students	21	3.2 00.5						
Contrary (officient hyginess)	733	90.3						
	32	0.2						
Households $N = 352$	n	%						
Average persons/household, median; [range]	5; [1; 11]							
Bed net availability								
No net	270	76.7						
1-2 persons/net	21	6.0						
\geq 3 persons/net	61	17.3						
Type of roof								
Aluminium	330	93.7						
Others (leaves, tiles, wood, bamboo)	22	6.3						
Type of wall								
Wood	310	88.1						
Bamboo	41	11.6						
Brick	1	0.3						
Cinnamon plantation								
Owns cinnamon plantation	332	94.3						
Average walking distance (hours) from village to plantation,								
median; [range]	1h. [3 min; 8 hr]							
Economic status								
Lowest income	219	62.2						
Low	27	7.7						
Higher	106	30.1						

Table 1. Baseline characteristics of the study population

field work (adults). Malaria prevalence was 7.8% (113/1,450) by LM, ranging between the four villages from 3.9 to 10.9%, and from 0 to 41.2% across the 20 clusters (Figure 1). Malaria prevalence was the highest (13.9%) in the ten to 19 years old children, except for Village 4 where the highest prevalence (15.4%) was found in younger children (<ten years).

Overall *P. falciparum* mono-infections represented the majority of LM detected infections (81.4%) although such proportion varied significantly between villages, i.e., from 59% in Village 1 to 96% in Village 3. The mean parasite density was significantly higher in *P. falciparum* (2,006.9/µl) than

in *P. vivax* (559.7/µl) infections, and increased with increasing prevalence of infection. Most infections were asymptomatic (69.9%), and approximately one-third (36.3%) carried gametocytes, with significant differences between villages (p <0.001). The prevalence of gametocyte carriage by village was significantly correlated to the prevalence of *P. vivax* infections (R^2 =0.99; p=0.002). Overall, the risk of gametocyte carriage was significantly higher in asymptomatic (46.8%) than in symptomatic (11.8%) infections (p <0.001), and in *P. vivax* (95.0%) compared to *P. falciparum* (22.8%) infections (p

<0.001). In addition, the risk of asymptomatic infection was significantly higher among *P. vivax* than *P. falciparum* infections (90 vs 65%, p=0.027).

A total of 327 individuals were randomly selected to estimate malaria prevalence by PCR; their socio-demographic characteristics were similar to those of the whole population. Malaria prevalence estimated by PCR was about three-fold higher than by microscopy (23.6 *versus* 7.8%), ranging by village between 16.4 and 42.5% (Table 2) and by cluster between 0 and 66.7% (Figure 1). The proportion of *P. vivax* mono-infections was higher when determined by molecular methods than by LM (39.6%, 95% CI [28.7; 51.6] *vs* 17.7%, 95% CI [11.6; 26.1]), while mixed species infections remained scarce (n=1). More than half (58.7%) of all infections were subpatent, i.e., negative by LM, and this proportion increased with decreasing village prevalence of infection as shown by the evolution of the ratio between sub-patent and patent infections (Figure 2A). The latter was strongly correlated with the ratio *P. vivax/P. falciparum* (Pv/Pf; R²=0.996; p=0.002).

Malaria prevalence significantly decreased with increasing age (p=0.007), from 32.3% in children <ten years to 15.4% in adults \geq 20 years, mainly

	Village 1	Village 2	Village 3 Village 4		TOTAL		
	% (n)				% (n/N)	[95% CI]	
Total screened (by microscopy)	350	256	532	312	1,450		
Parasite prevalence (microscopy)	4.9 (17)	3.9 (10)	9.8 (52)	10.9 (34)	7.8 (113/1450)	[6.3; 9.6]°	
Prevalence by age group (years):							
≤9	4.7 (6/127)	1.2 (1/84)	9.9 (20/202)	15.4 (18/117)	8.5 (45/530)	[6.3; 11.3] °°	
10-19	7.6 (5/66)	7.6 (4/53)	25.6 (20/78)	11.4 (8/70)	13.9	[9.4; 20.0]	
20-39	4.2 (4/95	5.3 (4/75)	5.6 (9/160)	7.9 (6/76)	(37/267)	[3.8; 8.3]	
\geq 40	3.2 (2/62)	2.3 (1/44)	3.3 (3/92)	4.1 (2/49)	5.7 (23/406)	[1.6; 6.4]	
					3.2 (8/247)		
Species distribution (proportion):							
- P. falciparum	58.8 (10)	80.0 (8)	96.2 (50)	70.6 (24)	81.4 (92)	[72.8; 87.8]°	
- P. vivax	41.2 (7)	20.0 (2)	1.9 (1)	29.4 (10)	17.7 (20)	[11.6; 26.1]	
- Mixed	0	0	1.9 (1)	0	0.9 (1)	[0.1; 6.3]	
Parasite density (geometric mean):							
- P. falciparum	1390.9	938.5	2008.3	3007.6	2006.9	[1,523.8; 2,643.1]	
- P. vivax	340.9	423.3	726.6	794.9	559.7	[301.9; 1,037.7]	
Infections with gametocytes, % (n)	64.7 (11/17)	40.0 (4/10)	15.4 (8/52)	52.9 (18/34)	36.3 (41/113)	[28.0; 45.5]°°	
Proportion asymptomatic, % (n)	76.5 (13/17)	80.0 (8/10)	73.1 (38/52)	58.8 (20/34)	69.9 (79/113)	[60.1; 78.2]	
Total surveyed (PCR)	N=75	N=55	N=133	N=64	N=327		

 Table 2. Malariometric indices by study village (determined by microscopy and PCR)

Parasite prevalence*, % (n)	16.4 (13/75)	18.9	20.2	42.5 (24/64)	23.6	[19.0; 28.9]
		(12/55)	(25/133)		(74/327)	
Species distribution:						
P. falciparum (Pf)	25.8 (4)	52.6 (6)	77.5 (17)	58.6 (14)	59.2 (41)	[47.2; 70.2]°
P. vivax (Pv)	74.2 (9)	47.4 (6)	22.5 (8)	37.9 (9)	39.6 (32)	[28.7; 51.6]
Pf + Pv	0	0	0	3.5 (1)	1.2 (1)	[0.2; 8.7]
Prop. sub-patent infections, % (n)	79.0 (10)	73.7 (8)	44.9 (14)	57.2 (14)	58.7 (46)	[46.2; 70.2]
Prop. asymptomatic infections, %	100.0 (13)	71.9 (9)	62.3 (15)	78.6 (18)	75.4 (55)	[63.7; 84.2]
(n)						
Prevalence ratio LM/PCR	0.30	0.21	0.48	0.26	0.33	
Mean haemoglobin, g/dl	13.1	13.4	11.9	11.9	12.4	[12.2; 12.6]**
Prevalence anaemia (Hb <10.0 g/dl)	3.4 (2)	2.7 (1)	14.9 (19)	11.7 (7)	9.6 (29)	[6.6; 13.7]°

because of a four-fold reduction of the prevalence of patent infections (from 16.0 to 4.0%; p <0.05) with increasing age. Sub-patent infections did not decrease significantly with age (16.4 to 11.4%, p=0.5), resulting in higher ratio sub-patent/patent infections in adults (Figure 2C). Similarly, the evolution of the ratio sub-patent/patent infections by age was also strongly correlated to the ratio *P. vivax/P. falciparum* infections which increased from 0.6 in the youngest age group to 1.14 in adults (R²=0.996; p=0.014) (Figure 2D). Overall, *P.vivax* infections were more likely to be sub-patent (86.2 *vs* 39.5%; p <0.001) and asymptomatic (63.5 *vs* 92.5%; p=0.02) compared to *P. falciparum*.

A total of 304 filter papers were available for the analysis of the G6PD Viangchan mutation and 297 were successfully genotyped. The estimated allelic frequency was 1.3% (2/154) among males and 1.4% (4/143) among females, and this was similar in Cadong (respectively 0 and 1.6%; p=0.4) and M'nong ethnic groups (1.5-1.4%; p=0.5).

Village, age, ethnicity, bed net ownership, occupation, and wall type were risk factors for malaria infection identified by the univariate analysis



Figure 2. A) Ratio sub-patent/patent malaria infections according to malaria prevalence by village; B) Ratio sub-patent/patent malaria infections according species prevalence by village and ratio Pv/Pf; C) Ratio sub-patent infections according to prevalence by age group; D) Ratio sub-/patent according to species prevalence by age group and ratio Pv/Pf

(Table 3); the multivariate adjusted analysis confirmed that only village, bed net and wall type were independently associated with malaria infection. Indeed the odds of malaria infection were almost four times higher in Village 4 compared to Village 1 (AOR=3.49); bed nets had a significantly protective effect (AOR=0.44), while people living in wooden houses were more likely to be infected as compared to those, though few, living in bamboo houses.

In order to overcome the difficulty of handling the interaction (age/villages) and multiple collinearities (ethnicity/village, age/occupation), the CART method was also used to identify and rank the main risk factors for malaria infections (Figure 3). The results showed that the first splitter was age (analysed as a continuous variable), individuals less than 20 years old being the most infected (30.3%) while adults (\geq 20 years old) were half as infected (16.5%). Among children the risk of infection was much higher in Villages 3 and 4 (37.9%) compared to Villages 1 and 2 (19%), while among adults the risk was highest in Villages 2 and 4 (25.8%) compared to Villages 1 and 3 (11.7%). In the high-risk villages, and in both arms of the tree, individuals

Cross-sectional survey N = 327	n/N	%	95% CI	OR	95%CI	AOR	95% CI
Overall	74/327	22.6	[18.3; 27.6]			_	
Village						_	
Village 1	13/75	16.4	[9.4; 27.0]	1		1	
Village 2	12/55	18.9	[10.2; 32.0]	1.18	[0.45; 3.11]	1.46	[0.54; 3.92]
Village 3	25/133	20.2	[13.7; 29.0]	1.29	[0.58; 2.85]	1.49	[0.66; 3.35]
Village 4	24/64	42.5	[30.3; 56.0]	3.77*	[1.64; 8.66]	3.49*	[1.51; 8.03]
Ethnic							
Cadong	24/64	42.5	[30.3; 56.0]	1			
M'nong	50/263	18.9	[14.3; 25.0]	0.31*	[0.17; 0.59]	_	
Age groups, years							
≤9	26/82	32.3	[22.9; 43.0]	1			
10-19	18/63	30.7	[20.0; 44.0]	0.93	[0.44; 1.96]		
20 +	30/182	15.4	[10.6; 22.0]	0.38*	[0.20; 0.72]	_	
Occupation							
None (children, disabled)	15/51	30.1	[18.9; 44.0]	1			
Farmers	33/192	16.5	[11.6; 23.0]	0.46*	[0.22; 0.96]		
Others (officer, business,							
students)	26/84	32.8	[23.2; 44.0]	1.13	[0.52; 2.48]	_	
Bed net availability in household							
No net	63/254	26.7	[21.3; 33.0]	1		1	
At least one net	11/73	12.7	[6.8; 22.0]	0.40*	[0.19; 0.84]	0.44*	[0.20; 0.97]
Wall of house							
Bamboo wall	3/37	6.1	[1.7; 19.0]	1		1	
Wood wall	71/290	25.7	[20.7; 31.0]	5.30*	[1.40; 20.06]	5.71*	[1.47; 22.1]

 Table 3. Multivariate adjusted risk factor analysis for malaria infections detected by PCR, using survey logistic regression

CI: Confident interval; OR: Odd ratio; AOR: Adjusted odd ratio; *p<0.05

living in wooden houses were much more infected compared to those in bamboo houses, except for those owning at least one bed net. The overall ranking showed that village and bed net ownership were the most important variables associated with the risk of malaria infection.

The CART analysis for the risk of patent infections (n=113) using the screening dataset (N=1,450) showed that the first splitter was village, i.e. Villages 3 and 4 with the highest prevalence, and in these villages children had the highest risk of infection (Figure 4). The ranking showed that village and age were the most important variables, while walls, ethnicity, income, education, and gender were not associated with the risk of the patent infection.

The multivariate adjusted model for the risk of patent infections confirmed that village 3-4, young age, and absence of bed nets in the house were significant risk factors though the output was not shown as the model could not handle multiple collinearities (ethnic/village, age/occupation) and the interaction (village*age).



Figure 3. Categorical tree showing the main risk factors for all malaria infections detected by PCR (n=327)

Finally, assessing simultaneously the risk of patent and sub-patent malaria infections with CART, age was the only splitter, as adults had a lower risk than children particularly for patent (4.4 *vs* 13.8%, respectively) while the difference was less important for sub-patents infections (12.1 *vs* 16.6%).



Figure 4. Categorical tree showing the main risk factors for patent malaria infections (detected by microscopy and PCR)

Discussion

In this remote, hilly and forested area populated by impoverished local ethnic minorities living from slash-and-burn farming, prevalence of malaria infection at the beginning of the rainy season (April) was extremely heterogeneous, both by LM and PCR, although the latter was performed only in a subset of the population, resulting in a lower precision. Heterogeneity of malaria transmission within short distances is a well-known, albeit not fully understood, phenomenon [19] but recently accumulated evidence showed that the identification and targeting of malaria "hotspots" (geographically limited areas with increased transmission and asymptomatic parasite carriage) are key to efficient elimination efforts [20, 21]. Identifying parasite carriers, especially those with sub-patent infections, represents a challenge as it requires the use of more sensitive diagnostic (molecular, serological) tools in large scale population surveys. Our study area is a typical example of the remaining foci of malaria transmission (including hotspot(s)) in forested areas of Central Vietnam, and illustrates the challenges for malaria elimination.

Subpatent infections in this area represented an important proportion of all detected infections, with the difference between LM and PCR estimates similar to other reports [8, 22-24] and an overall ratio LM/PCR prevalence (0.33) which corresponded well to the predicted PCR prevalence band (10-24%) reported by a systematic review that included 72 pairs of prevalence estimates across the three continents [23].

Gametocyte carriage as determined by LM was similar to that found in other surveys carried out in Central Vietnam [8, 25]. When considering that less than 10% of gametocyte carriers are actually detected by microscopy [23], gametocyte carriage in this study population would be almost 25%, indicating that even in this area of low transmission the infectious human malaria reservoir is substantial and largely hidden, and consequently challenging current control strategies largely based on passive case detection of malaria cases by LM [26]. Although the contribution of individuals with subpatent gametocytaemia to malaria transmission remains unclear, it has recently been shown that the relationship between gametocyte density and infectiousness to mosquitoes is highly non-linear [27]. In Kenya and Burkina Faso, although individuals with very low gametocyte densities (from less than 1 gametocyte/µl to 200/µl) were infectious to only 4% of all mosquitoes, such proportion raised rapidly at densities between 200-400/µl, to reach a plateau at 18% of all infected mosquitoes. Similarly, even though children in Burkina Faso had much higher gametocyte densities compared to adults, the latter still contributed largely to malaria transmission on the basis of their number and on the occurrence of sub-patent infections in this age group.

The proportion of gametocyte carriers as determined by LM varied by village, and overall was highly correlated with the proportion of *P. vivax* infections, possibly due to the ability of this species to produce gametocytes at very early stage of its erythrocytic schizogony, well before the occurrence of symptoms [28-30]. The fact that the large majority of *P. vivax* infections were also asymptomatic and subpatent can explain the association between gametocyte carriage and asymptomatic infections.

Unlike a previous study carried out in the neighbouring Ninh Thuan Province [8], *P. malariae, P. ovale* or mixed infection were not common, reflecting the high heterogeneity of malaria transmission and species distribution. In this case, the absence of these species could be explained by the higher isolation and remoteness of the four study villages compared to those in Ninh Thuan Province where some of the villages were located near the district town or along the main district road, favouring parasite strains and species circulation through population movements to different endemic areas.

Malaria risk was the highest in people living in Village 4; because of colinearity, such risk could not be dissociated in the logistic regression model from Cadong ethnicity since these were all living in Village 4. Indeed, ethnicity has been repeatedly reported as being associated with malaria infection mainly due to socio-cultural [31-34] or to genetic factors [30, 31, 35]. The analysis of the G6PD polymorphism, at least for the most common Viangchan mutation, did not show differences between the two ethnic groups, though further analysis of G6PD and other genetic polymorphisms would help in investigating potential associations between genetic polymorphisms, ethnicity and malaria risk as shown in other settings [29, 30]. From the investigators experience as well as anthropological expert opinion (Koen Peeters, personal communication) there were no intrinsic socio-cultural differences between Cadong and M'nong ethnic groups. An alternative explanation for the higher risk of malaria in Village 4 could be the fact that rice fields in this village were situated much further (2-3h walking) from people's houses as compared to the other three villages (15-30min walk). Consequently, during the months of harvest (July-October) and field preparations (February-April) farmers in Village 4 used to stay for prolonged periods with their families in their forest fields (plot huts) where they were more exposed to mosquito bites.

Further analysis by CART showed that village but not ethnicity was the most important risk factor both for PCR detected malaria infections as well as for patent infections. Age was ranked second in importance for the risk of patent infections, but not for the risk of all infections detected by PCR; this may reflect the progressive build-up of partial immunity with age as illustrated in Figure 2. Similar results were found in previous report from Ninh Thuan Province where the ratio of sub-patent infections significantly increased from less than one in children below 20 years to around two in adults [8].

Occupants of wooden houses were at higher risk of malaria infection. These were the most common type of houses and had been built by the government, thus not reflecting the actual socio-economic status of respective dwellers. Bamboo houses, usually with smaller doors and windows compared to wooden houses, are traditional for these ethnic minorities and may result in a lower risk of exposure to mosquito bites, though sampling variation cannot be excluded given the very small number of bamboo houses. Conversely, availability of bed nets, even non-treated, was protective, particularly in wooden houses, and was ranked as the second most important risk factor for malaria infection.

Conclusions

The malaria situation in this study area reflects the difficulties related to the goal of malaria elimination in Vietnam. There was a substantial and hidden

human reservoir of malaria infection, largely represented by *P. vivax*. The remoteness of the area together with the difficulty of detecting and treating both sub-patent and patent infections, particularly those carrying gametocytes, and *P. vivax* liver forms that keep releasing parasites in the blood stream, represent huge challenges for any malaria elimination programme. Until new approaches for dealing with these issues are available, eliminating malaria in these types of settings will remain extremely challenging.

Abbreviations

PCR: polymerase chain reaction; G6PD: glucose-6-phosphate dehydrogenase; CART: classification and regression tree method; CHC: Commune Health Centre; VHWs: village health workers; LM: light microscopy; FPBS: filter paper blood samples; DNA: deoxyribonucleic acid; WBCs: white blood cells; NIMPE: National Institute of Malariology, Parasitology and Entomology.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

PVT was involved in all phases of the study, study design, data collection, data analysis, and writing of the manuscript; NVH did all PCR analyses; NVV

was involved in data collection and monitoring of field activities; GB and NC analysed samples for G6PD mutations; CM contributed to data analysis; VO produced the map of the study site; AR, TTD and UD contributed to data analysis and critical revision of the manuscript; KPG and NXX designed questionnaires; NS supervised data analysis; AE was involved in all phases from study design to revisions of the manuscript. All authors read and approved the final version of the manuscript.

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CHAPTER IV

Confirmed *Plasmodium vivax* resistance to chloroquine in Central Vietnam

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Abstract

Plasmodium vivax resistance to chloroquine (PvCOR) is currently reported in almost all vivax endemic countries. In Vietnam, despite a first report on PvCQR published in the early 2000s, P.vivax was still considered sensitive to CQ. Between May 2009 and December 2011, a 2-year cohort study was conducted in Central Vietnam to assess the recommended radical cure regimen based on a 10-day course Primaquine (0.5mg/kg/day) together with 3 days CQ (25mg/kg). We hereby report the results of the first 28-day followup estimating the cumulative risk of P. vivax recurrences together with the corresponding CQ blood concentrations among other endpoints. Out of 260 recruited *P.vivax* patients, 240 completed treatment and were followed up to day 28 according to the WHO guidelines. Eight patients (3.45%) had a *P.vivax* recurrent infection, at day 14 (n=2), day 21 (n=1) and day 28 (n=5). Chloroquine blood concentrations, available in 3/8 recurrent infections (day 14,21,28) were above the minimal inhibitory concentration (>100ng/ml whole blood) in all of them. Fever and parasitaemia (both sexual and asexual stages) were cleared by day 3. Anemia was common at day 0 (35.8%) especially in children below 10 (50%) and hemoglobin (Hb) recovery at day 28 was substantial among anemic patients (median change d28-d0 = +1.7g/dl; IQR[+0.7; +3.2]). This report, based on CQ blood levels measured at the time of recurrences, confirms for the first time P. vivax CQ resistance in Central Vietnam, and calls for further studies using standardized protocols for accurately monitoring the extent and evolution of PvCQR in Vietnam. These results, together with the mounting evidence of artemisinin resistance in Central Vietnam, further highlight the increasing threat of antimalarial drug resistance on malaria elimination in Vietnam.

Key words: Plasmodium vivax, chloroquine, resistance, Central Vietnam

Introduction

Plasmodium vivax is the most worldwide distributed malaria parasite species with an estimated 2.85 billion people at risk of infection in 2009, the vast majority (2.59 billion, 91.0%) living in Central and South East Asia [1]. Moreover, since malaria elimination has been set on the global health agenda [2], the public health importance of vivax malaria is increasingly reassessed since it is more difficult to control than P. falciparum, severe clinical syndromes as well as new foci of chloroquine resistance are increasingly reported [3-5]. Chloroquine (CQ) is the first-line treatment for P. vivax in most endemic countries. P.vivax resistance to CQ (PvCQR) was first reported in 1989 from Papua New Guinea [6], rapidly followed by reports from Indonesia in 1991 [7, 8], Myanmar in 1993 and 1995 [9, 10], India in 1995 [11, 12], Malaysian Borneo in 1996 [13], and in several South American countries (Guyana, Brazil, Columbia) from 1996 onwards [14-16]. In Vietnam, little evidence on P. vivax susceptibility to CQ has been published so far: one study in Binh Thuan province (Southeastern coast region) in the early 2000s reported PvCQR [17], while this was absent in the neighboring Khanh Hoa province [18]. The national malaria control program (NMCP) has been closely monitoring antimalarial drug resistance, mainly focused on P. falciparum resistance [19-21], since 1995 in several sentinel sites across the country. Since 2003, *P. vivax* susceptibility to CQ has been assessed in six sentinel sites and reported between 0-5.7% of late parasitological failures [22].

Vietnam is currently engaged in malaria elimination [23, 24] and the issue of drug resistance is a priority as P. falciparum resistance to artemisinins has been already reported in five (Tier I) provinces of Central Vietnam [25, 26]. Moreover, the control of *P. vivax* is another challenge as this species is becoming increasingly prevalent [27-30]. The main difficulty in controlling vivax malaria lies in the need of radically treating not only blood forms but also the hepatic dormant forms (hypnozoites) that cause relapses for the next months/years after the initial infection. The World Health Organization (WHO) currently recommends for radical cure a 3-day course of CQ (total 25mg/kg) together with a 14-day course of primaquine (PQ, 0.25mg/kg/day), the recommended treatment in Vietnam since 2009. Nevertheless, between 2007 and 2009, instead of the 14-day course, PQ was given for 10 days at a higher dose (0.5mg/kg/day) [31]. The efficacy of such treatment on liver stages was assessed by following up in Central Vietnam a cohort of P. vivax treated patients for two years. We report here the results of the first 28-day follow-up done according to the WHO guidelines [32].

Methods

Study site and participants

The study was carried out between April 2009 and December 2011 at the Tra Leng Commune Health Center (CHC), located in a remote forested area in the southwestern part of Quang Nam province, Central Vietnam. A detailed description of the study area and population has been reported elsewhere [33]. The study was designed as a 28-day follow-up after treatment of *Plasmodium* vivax cases with CQ and PQ [32]. Male and female patients, aged between 3 and 60 years, presenting at the CHC (or identified through active case detection by the study team) with suspected malaria were screened for eligibility. Inclusion criteria were: axillary temperature ≥37.5°C and/or history of fever in the previous 48 hours, P. vivax mono-infection with asexual parasites confirmed by light microscopy (LM), residency in the study area, and written informed consent from all participants aged 18 years or older (parents/guardians for minors). Patients were excluded if they presented general danger signs with severe or complicated malaria, any acute or chronic concomitant illness, or if they had already been treated with PQ within the past 30 days. Pregnant or lactating women, patients with known G6PD deficiency (or history of "black urine" following PQ treatment), or any history of intolerance the study drugs were excluded. According to the national guidelines, patients were not tested for G6PD deficiency prior to PQ treatment. The prevalence of G6PD genetic polymorphism (*Viangchan* mutation) was estimated to be below 1.5% in both males and females [33] with no difference between ethnic groups.

Procedures

Study drugs were provided by the national malaria control program and consisted of CQ tablets of 300mg chloroquine base (Lot No: 08001CN; Registration number: VNB-4144-05) and PQ tablets containing 15mg primaquine base (Lot No: 010109; Registration number: VD-0877-06).

A general physical examination was performed at inclusion (day 0), and daily during treatment (days 1 to 9); subsequently, patients were examined weekly at days 14, 21, and 28, and during any unscheduled visit. Patients were asked to return daily at the CHC for direct observed therapy with CQ (25mg base/kg) and PQ (0.5mg/kg/day) during the first three days (day 0-2) and then with PQ alone for the remaining 7 days (days 3-9). More specifically, signs and symptoms of acute hemolysis (jaundice, black urine, fatigue, tachycardia, shock...) were systematically checked at each visit by the study clinician; adverse drug reactions and concomitant medications were recorded. Patients not attending scheduled visits were visited at home. Any recurrent *P. vivax* or *P. falciparum* infection detected by LM during the 28-day follow-up was treated with dihydroartemisinin-piperaquine (DHA-PPQ) for 3 days following national guidelines.

Blood samples (finger prick) were collected at days 0, 1, 2, 3, 7, 14, 21, and 28 for LM (blood films) and later molecular and analysis (2 blood spots dried on filter paper). Additional blood samples were taken at day 0, 14 and 28 for hemoglobin concentration; at day 7 and any day of recurrent *P.vivax* infection, 100μ l of blood were taken on a separate filter paper for later measurement of CQ blood level.

Thick and thin films were stained with 3% Giemsa solution for 45 minutes; parasite density was estimated by counting the number of parasites per 200 white blood cells (WBCs) and assuming 8.000 WBC/µl. A slide was declared negative if no parasite was found after counting 1,000 WBCs. All slides were read independently by two expert technicians who in case of discrepancy reread the slide until reaching agreement. A later and systematic quality control of all blood slides was done by a senior technician at central level (NIMPE Hanoi); in case of disagreement, a second senior technician would re-read the slide until agreement. Hemoglobin concentration was measured with the

HemoCue Hb 301 (HemoCue AB, Angelholm, Sweden) device following the manufacturer instructions [34]. Filter paper blood samples (FPBS) were dried outside direct sunlight, kept in individual sealed plastic bags, and stored at - 20°C (NIMPE, Hanoi) until they were processed.

The concentrations of CQ and desethylchloroquine (DEC) in blood-dried filter paper were determined using a validated HPLC method with fluorescence detector at Excitation/Emmision 250/400 nm, a modification from previous published method [35]. Following mincing of the filter paper (Whatman grade 3), extraction was performed using 3 mL of 25% ammonia and 3 mL of ethyl acetate-hexane (1:9). The solution was vortexed for 30 s and centrifuged to separate the organic phase which was then transferred to another tube and evaporated to dryness. The sample was reconstituted with HPLC mobile phase and 20 μ L was injected to the HPLC system (Waters, USA). We used X-Bridge Phenyl 5 μ m (4.6 x 150 mm) column as stationary phase. The mobile phase used was diethylamine 0.05%: acetonitrile (55:45), pumped isocratically at flow rate of 1.0 mL/min and temperature of 30°C. Pyrimethamine was used as internal standard.

Outcomes

Efficacy outcomes were classified into early treatment failure (ETF), late clinical (LCF) or parasitological failure (LPF), or adequate clinical and parasitological response (ACPR), following the WHO criteria [32]. For all efficacy outcomes, no distinction was made between relapse, recrudescence and re-infection, and any new microscopically detected *P. vivax* infection after initial parasite clearance was defined as "*P. vivax* recurrence". The primary endpoints were the proportion of patients with ACPR by day 28 and the parasite clearance time (PCT). Secondary endpoints included fever and gametocyte clearance times, the proportion of confirmed CQ resistant *P. vivax* recurrences (CQ +DEC >100ng/ml), and hematological changes between days 0 and 28.

Data Analysis

The sample size was calculated on the basis of retrospective data (2003-2007) reporting LPF among *P. vivax* patients treated with CQ ranging from 0% to 5.7% [22]. Assuming a minimum treatment failure rate of 5% and a loss to follow up of 10%, 204 vivax patients would be able to estimate it with a 3% precision and at 5% significance level ("CSample" command/Epi Info 6). The sample size was further increased to comply with the requirements of the cohort evaluation for which details will be published separately. Data were

double entered and cleaned using Epidata version 3.1. The data set was analyzed using the STATA version 11 (Stata Corp, College Station, TX). The survey design (survey dataset) was taken into account using the svycommand in STATA, with villages as strata, and household as sampling unit. Descriptive statistics were used to compute baseline socio-demographic characteristics. Ownership of livestock (pigs, buffaloes, and cows) was used as a proxy for the economic status of the household using a principal component analysis [33]. The PCT was estimated using the daily proportion of patients still parasitemic from day 0 until the day of complete parasite clearance. The proportion of recurrence-free patients by day 28 was assessed by Kaplan Meier survival analysis. Patients were censored on the day they had last been seen in follow-up. Fever clearance time was estimated by determining the proportion of febrile patients during follow up among febrile patients at day 0. Similarly, gametocyte clearance was expressed as the proportion of patients with gametocytes during follow up among gametocyte positive patients at day 0. Hematological recovery was estimated by computing the median Hb concentration at day 0, 14 and day 28 as well as the median of individual Hb differences between day 0 and day 28. Anaemia was defined as Hb<11g/dl, for both sex and all ages [36]. The Wilcoxon rank sum test and Sign rank test were applied as required to compare Hb medians. A survey logistic regression ("svy" command in STATA) was used to carry out a multivariate adjusted analysis for the risk of anaemia before and after treatment (adjusting for all potential confounders such as sex, age, baseline parasitemia, splenomegaly and ethnicity). Similarly, survey logistic regression was also used to assess if baseline parasite density (day 0) or age were independently associated with parasite clearance at day 2. A multivariate linear regression model was used to determine the independent effect of the baseline Hb values (day 0=Hb0) on the relative Hb changes at day 14. Potential risk factors (age, ethnicity,...) with a p-value of <0.05 in the univariate analysis were included in the multivariate model and retained if the p-value was <0.05. (age, anemia at day0, ethnic, ...). Interactions were systematically checked for up to order two. The 5% cut off was defined as a significant p-value for all statistical tests.

Ethical clearance

Ethical clearance was obtained from both the Ethical Committee of the NIMPE in Hanoi and of the University of Antwerp. The fundamental principles of ethics in research on human participants were upheld throughout the project. The study objectives and methods were explained to the people's committee, village's leader and the local people. All study participants had
given their informed consent after being explained the study objectives and procedures as well as their right to withdraw without prejudice for themselves or their families. Written informed consent was obtained from parents or guardians of children below 18 years; children between the age of 12 and 18 years were asked to provide a written assent.



Figure 1. Study profile

Results

Trial profile and baseline characteristics

Between April 2009 and December 2010, 260 *P. vivax* infected patients were enrolled and given the 10-day radical treatment of PQ (0.50mg/kg/day) associated with CQ for the first three days (total 25mg/kg); 240 patients (92.3%; 240/260) completed the treatment and were included in the analysis, and 232 patients completed the 28-day follow-up. All incomplete follow-ups were due to consent withdrawal (Figure 1) following prolonged absence, mainly because of work requirement in forest fields. Patients were recruited in all four study villages and the vast majority (78.5%; 204/260) belonged to the M'nong group (Table 1). Males (61.1%; 159/260) slightly outnumbered females, and almost half of the participants (43.1%; 112/260) were children aged 3 to 9 years. The majority of participants had no bed net at home (70.8%; 184/260), their socio-economic status was very low, and all adults were farmers.

More than half of the study patients (59.2%; 154/260) had measurable fever at enrolment; headache (36.1%; 94/260) and fatigue (33.1%; 86/260) were the most common symptoms, and about 6% (16/260) had an enlarged spleen. The mean parasite density at enrolment was $2,754.1/\mu$ l (geometric mean=GM) and gametocytes were found in most of the patients (86.1%; 224/260) though at much lower densities (GM=387.7/ μ l). The median hemoglobin at enrolment was 11.7g/dl and more than one third of the patients (35.77%; 93/260) were anaemic (Hb<11g/dl). The treatment was well tolerated, no clinical sign or symptoms of acute hemolysis were observed (despite the occurrence of transient acute hemolysis, see below) and only few patients (12.3%; 32/260) complained of nausea following PQ administration though none of them vomited their dose of CQ or PQ.

Table	1.	Baseline	demographic,	clinical	and	parasitological	
characteristics at enrolment							

(N=260)	n	%	95%CI
Village			
Village 1	101	38.85	[34.83; 43.02]
Village 2	64	24.62	[21.25; 28.33]
Village 3	39	15.0	[12.84; 17.45]
Village 4	56	21.54	[17.63; 26.04]
Gender			
Male	159	61.15	[55.76; 66.82]
Female	101	38.85	[33.72; 44.24]
Ethnic groups			
M'nong	204	78.46	[73.96; 82.37]
Cadong	56	21.54	[17.63; 26.04]
Age groups (years)			
3-9	112	43.08	[37.45; 48.89]
10—19	71	27.31	[21.84; 33.55]
20—29	44	16.92	[12.75; 22.11]
30—60	33	12.69	[9.36; 17.0]
Occupation			L / J
No (children < 6 years old)	70	26.92	[22.02; 32.47]
Farmer	85	32.69	[27.51; 38.34]
Pupil	105	40.38	[34.34: 46.74]
Bednet in house			[- ··· , ···]
No	184	70.77	[61.81; 78.37]
At least one	76	29.23	[21.63; 38.19]
Economic status ^o			. , ,
Lowest income	147	56.54	[47.20; 65.43]
Low	26	10.0	[5.70; 16.96]
Higher	87	33.46	[25.18; 42.9]
Clinical symptoms (most frequently reported)			
Fever (axillary temperature ≥ 37.5)	154	59.23	[53.06; 65.12]
Headache	94	36,15	[30.42; 42.32]
Fatigue	86	33.08	[27.23: 39.5]
Dizziness	28	10.77	[7.51: 15.22]
Nausea	32	12.31	[8.83: 16.90]
Enlarged spleen	16	6 1 5	$[3.58 \cdot 10.39]$
Laboratory data		0,10	[5:50, 10:57]
Asexual parasite density/ul. GM [95%CI]	2754.07 [2271.87: 3338.61]		
Gametocyte density/ul. GM [95%CI]	3	87.72 [32	4.84: 462.80]
Patients with gametocytes	224	86.15	[81.37: 89 86]
Hemoglobin (g/dl: Median: IOR)	·	11.7 [1	0.4: 13.1]
Patients with anemia (Hb<11g/dl)	93	35.77	[29.8: 42.21]
- whome with anothing (110 -115/41)	15	22.11	[27.0, 12.21]

^o Score in tertiles defined as "high", "medium", and "low" economic status, following principal component analysis. GM: Geometric mean; IQR: Inter-quartile range

Primary endpoints

No ETF was observed; there were eight late treatment failures, 2 LPFs at day 14, 1 LCF at day 21, and 5 LPFs at day 28 (Table 2). ACPR at day 28 was 96.6% (95%CI [93.7; 98.2]). *P. vivax* recurrence was not associated with delayed parasite clearance as five of the eight patients with recurrence had cleared parasitemia before 24h. The mean parasite density at day of recurrence was very low (GM=41.1/ μ l; IQR [23.3; 855.8]).

At day 1, more than half of the patients (57.9%) were still parasitaemic, at day 2 they were only 7.1%, and at day 3 none of them had detectable parasitaemia. Parasite clearance at day 2 was significantly associated with a higher asexual parasite density at day 0 (OR=1.79; 95%CI [1.14; 2.82]; p=0.012) but not with age.

Primary endpoints (N = 240)	n (%)	95%CI			
Adequate clinical and parasitological response (KM*)	224 (96.55)	[93.67; 98.15]			
Cumulative incidence of treatment failures (KM)	8 (3.45)	[1.85; 6.33]			
Late clinical failures (day 21)	1				
Late parasitological failures $(n = 7)$:					
Day 14	2				
Day 28	5				
Patients with asexual parasitemia:					
Day 1	139 (57.92)	[51.44; 64.13]			
Day 2	17 (7.08)	[4.39; 11.23]			
Day 3	0				
Secondary endpoints					
Fever clearance (n=139=100% at D0):					
Day 1	34 (24.46)	[18.7; 31.32]			
Day 2	5 (3.59)	[1.49; 8.41]			
Day 3	0				
Gametocytes clearance (n=207=100% at D0)					
Day 1	83 (40.1)	[33.96; 46.56]			
Day 2	11 (5.31)	[3.0; 9.23]			
Day 3	0				
CQ blood concentrations at day of failure >100ng/ml:	3/3				
Day 14 (LPF)	114.66				
Day 21 (LCF)	133,09				
Day 28 (LPF)	125,87				
Hemoglobin recovery, median individual Hb changes D0-28 (g/dl [IQR])					
All patients (n=224)	+0.7 [-0.2; +1.6]				
Anemic at D0 (n=78)	+1.7 [+0.7; +3.2]				
Non anemic at D0 (n=146)	+0.25 [-0.4; +1.0]				

Table 2. Primary and secondary endpoints

*KM: Kaplan Meir estimate. IQR: Interquartile range.

Secondary endpoints

All patients were afebrile and without gametocytaemia by day 3 (Table 2). Dried blood samples for measuring CQ blood concentrations were available (at day 7 and of recurrence) for 5 of the 8 patients with vivax recurrence and, among these, three had interpretable results. The CQ blood concentrations at day 7 ranged from 365.1 to 1347.1 ng/ml, confirming adequate drug absorption. The three CQ blood concentrations at time of recurrence were 114.7 ng/ml (day 14), 133.1 ng/ml (day 21), and 125.9 ng/ml (day 28), all of them above the 100ng/ml threshold, confirming CQ resistance.

The median Hb value at day 0 among patients with ACPR (n=224) was 11.7g/dl [IQR=10.5; 13.1] and children were significantly more at risk of anemia (50.0%, 49/98) than older patients (23.0%, 29/126) even after adjusting for baseline parasitemia (AOR=3.60, 95%CI [1.88; 6.88]; p<0.001). By day 28, the median Hb increased to 12.3g/dl [IQR=11.3; 13.4] and the median value of individual Hb changes between day 0 and day 28 was of +0.7g/dl [IQR=-0.2; +1.6]. Among anemic patients at day 0 (n=78), the median Hb was 9.9g/dl [IQR=8.3; 10.5] and significantly increased to 11.5g/dl [IQR=10.8; 12.1] by day 28 (Sign rank test p<0.001) with a median change of +1.7 g/dl [IQR=+0.7; +3.2] (Figure 2A). This change was slightly lower in children (median=1.5g/dl, IQR [0.7; 2.5]) than in adults (median=1.9; IQR[1.1; 4.2]; Wilcoxon rank-sum test p=0.08). After treatment, 24.5% (24/98) children and 8.7% (11/126) adults were still



* Median Hb Change, P-value < 0.001 (Sign rank test)



Relative Hb change on day 14 (%) by linear regression: *i*) anemia group (Coef β =-10.00; 95%CI [-12.81; -7.19]; p-value < 0.001); *ii*) non-anemia group (β =-6.46; 95%CI [-7.41; -5.51]; p-value < 0.001). A significant interaction was found between Hb change at day 14 and anemia status at day0 (interaction term β =-5.99; 95%CI [-8.87; -3.11]; P-value<0.001)

Figure 2. A) Median hemoglobin (Hb) concentration at day 0, 14 and 28 (n=224 patients with ACPR); B) Relative Hb change (between day 14 and day 0) according to baseline Hb values (cut-off for anemia Hb<11.0g/dl) (n=240)

anemic (AOR=3.5, 95%CI [1.7; 7.0]; p=0.001). Patients who were still anemic by day 28 were treated with hematinic drugs (ferrous sulfate and folic acid).

In order to better understand the relationship between Hb changes, age and baseline Hb values (Hb0), we plotted the individual changes at day 14 relative to day 0 (%, Figure 2B) as a function of Hb0 and carried out a multivariate linear regression analysis adjusting for the potential confounding effect of age. The final model showed that relative Hb changes at day 14 were independently (and negatively) associated with Hb0 (p<0.001) and age was not a confounder since it was only associated to the exposure and not to the outcome variable. Moreover, the linear regression model showed that the effect of Hb0 on relative Hb changes at day 14 was significantly different between anemic and non-anemic patients at day 0 (interaction term coefficient β =-5.99; p<0.001). Indeed, while in non-anemic patients the Hb decreased by 6.5% for every increase in Hb0 unit (β =-6.45; p<0.001), in the anemic group, the Hb increased by 10% for every decrease in Hb0 unit (β =-10.00; p<0.001). Interestingly, 9 patients experienced a more than 25% reduction in Hb by day 14, ranging from -58.2% to -32.8%, without any sign or symptom of hemolysis detected during the 28-day follow-up. All but one of these patients had normal Hb values at day0, and the majority (6/9) of them had recovered a normal Hb value by day 28.

Similar results were found for the association between Hb0 and relative Hb changes by day 28 with a significant interaction (interaction term β =p<0.001) and a slightly stronger effect of Hb0 among anemic patients (β =-14.00; p<0.001) and a smaller effect (β =-3.38%; p<0.001) in the non-anemic group (data no shown).

Discussion

This study confirms for the first time *P.vivax* CQ resistance (PvCQR) in Vietnam as three patients with recurrent vivax infections were found to have CQ blood concentrations above the minimum inhibitory concentration (100ng/ml of whole blood). Suspected PvCQR was observed from Binh Thuan province in Southern Vietnam in the late 90s, with 16% treatment failure after a 3-day course of CQ (25mg/kg) [17] but could not be confirmed as CQ blood concentrations were not available. Indeed, the latter is necessary [37] as recurrent infections could be the consequence of inadequate drug concentration due to sub-optimal drug quality and dosage or low intestinal absorption rather than CQ resistance. For this study, these factors can be

excluded as the day 7 CQ concentrations were within the optimal range, at least for the five patients with available results at day 7.

Since the first reports from Papua New Guinea (PNG) in 1989 [6, 8, 38, 39], PvCQR has rapidly reached unacceptably high levels in Indonesia and PNG, prompting the WHO to recommend artemisinin based combination therapies for *P.vivax* [40]. Moreover, a recent systematic review showed that PvCQR can be found in most vivax malaria endemic countries across continents [41]. The apparently low cumulative risk of recurrence by day 28 estimated in our study together with the absence of ETF suggest a low grade PvCQR when compared to other SEA countries, particularly Indonesia, where ETFs ranged from 6% to 24%, and 28-day recurrence rates from 18% to 100% [41]. Similarly, the recurrence rate may be considered negligible when compared to that (16%) observed in Binh Thuan province about 15 years ago [17]. Nevertheless, when considering that CQ was co-administered with high dose (0.5mg/kg/day) PQ, which has also an effect on *P.vivax* asexual blood stages [42, 43], the estimation of PvCQR provided here is probably much lower than its true prevalence. Indeed, in Indonesia adding PQ to CQ decreased the day-28 treatment failure from 78% to 15% (39). Therefore, our seemingly low grade PvCQR is the CQ failure when combined to high dose PQ, while the true failure related to CQ resistance is probably higher, possibly up to fivefold higher (39). Therefore, PvCQR in Quang Nam province is probably similar to that reported 15 years ago from Binh Thuan province [17]. As CQ (monotherapy) efficacy measured in 6 sentinel sites in Central and Southern Vietnam between 2006 and 2011 has been consistently at 100% [24], it is possible that PvCQR in Vietnam has not reached the high levels observed in PNG and Indonesia. Indeed, despite the lack of power, with sample sizes between 25 and 65 patients [24], far below the minimum of 75 recommended by the WHO [32], it is unlikely that high grade PvCQR would have been missed. Therefore, PvCQR was present in Central and Southern Vietnam since at least the late 90s and, unlike in PNG and Indonesia, it did not evolve to high grade levels. The most likely explanation for such difference could be the much lower CQ pressure as artemisinin derivatives have been used since the early 90s for the treatment of multidrug resistance *P. falciparum*.

When considering the timing of the observed recurrent infections, the two LPFs at day 14 are probably recrudescences as *P.vivax* infections recurring before day 16 are almost certainly due to a recrudescence from the primary infection [37]. Infections recurring later may be either recrudescences or relapses, with CQ resistant parasites if the CQ blood level is above the minimum inhibitory concentrations [37]. As this is an area of extremely low transmission, more than one infectious bite within one month interval is

unlikely though it cannot be excluded as farmers often stay overnight in their forest fields where they are at higher risk of exposure to the main vector *An. dirus* [44, 45]. Genotyping alone is usually of limited help to distinguish between recrudescence and relapse/new infection since relapses can occur with either the same or different clones [46].

Vivax malaria associated anemia was common and hematological recovery at day 28 depended on baseline Hb. Indeed, the more pronounced hematological recovery was observed among patients who were anemic before treatment. This observation is similar to a recent report from PNG [47]. In addition, young children were at a much higher risk of anemia than older patients, and this risk remained high after treatment, illustrating the importance of an efficacious radical treatment for *P.vivax* in children [48]. The linear regression model showed that age was indirectly associated with Hb changes only through its significant association with Hb0. Moreover, in anemic patients, the lowest Hb0 values corresponded to the more marked Hb increase during follow up; for the other patients, the highest the Hb0, the more marked was the Hb decrease during follow up. This inverse relation could be partly explained by the increased hemolytic risk in older red blood cells [49] and by the suppressive activity of hemozoin (digested Hb) on erythropoiesis [50]. It is possible that anemic patients were infected for longer periods and at day 0 had already reached their lowest Hb value. This would have resulted in a more robust bone marrow response compared to non-anemic, recently infected patients (47). Transient asymptomatic Hb reductions (\geq -50%) after PQ treatment, both as radical cure or single gametocytocidal dose, have been observed among G6PD deficient and non-deficient African children [51-53].

A quick *post-hoc* genotyping [54] was carried out to screen for the four most commonly reported G6PD mutations in Vietnam (*Vieng Chang, Canton, Union, Kaiping*) among the 9 patients who experienced >25% reduction in Hb by day 14 (together with 9 randomly selected control patients (no change in Hb)). Only one patient was found positive for the *Vieng Chan* mutation, i.e. a 26-year old male of Cadong ethnicity with a transient Hb decrease of 52.9% by day 14 (Hb0=14.0) followed by a full recovery at day 28 (Hb28=13.6g/dl). It is not possible to exclude, among these 9 patients, the presence of other G6PD variants (i.e. *Vietnam I, -II, Gaohe Gaozhou, Coimbra*, etc) also reported in different ethnic groups from Central Vietnam [55, 56]. This will be further investigated by carrying out an in-depth analysis of the G6PD genetic polymorphism in all 240 study patients in relation to their Hb changes Moreover, the observed transient but potentially life-threatening hemolysis (>50% Hb change at day 14) questions the national policy that currently does not recommend G6PD testing prior to radical PQ

treatment. To better estimate the risk of hemolysis linked to PQ use, there is the urgent need of determining the prevalence of the G6PD deficient phenotype together with the G6PD genetic polymorphisms among different ethnic minorities living in residual malaria endemic areas.

The main limitation of our study is the concomitant use of PQ and CQ which most likely resulted in a substantial underestimation of the true CQ-related cumulative risk of recurrence by day 28. As per WHO recommendation [32], PvCQR can only be accurately estimated by standard 28-day *in vivo* studies with CQ monotherapy, PQ being withheld until day 28. Strictly speaking, PvCQR could not be confirmed in all eight vivax recurrences as CQ blood levels results were available only for three patients. However, given the pharmacokinetics of CQ [37], it is likely that the other patients also had adequate CQ blood levels. In addition, the concomitant administration of PQ and its synergistic effect on asexual blood stages could also explain why no association was found between the PCT and the risk of recurrence, unlike the one reported in the recent meta-analysis by Price and colleagues [41]. For all these reasons, a new study has been initiated using CQ monotherapy to accurately determine its *in vivo* and *in vitro* efficacy on *P.vivax* infections.

Another limitation of our study is the 24-hourly sampling schedule which was not optimal for an accurate determination of the PCT. For future studies, 8 to 12 hourly sampling and a baseline parasite density of at least $250/\mu$ l are needed to accurately determine parasite clearance [32, 57].

Conclusion

In conclusion, this is the first confirmed evidence of PvCQR in Central Vietnam, an area where we recently reported *P. falciparum* resistance to artemisinins [25]. PvCQR should continue to be monitored in different sentinel sites of Central Vietnam, using standardized and sufficiently powered *in vivo* protocols with CQ monotherapy and PQ therapy delayed to day 28. Vietnam has committed for malaria elimination by 2030 and within this context antimalarial drug resistance, not only *P.falciparum* resistance to arteminins but also *P. vivax* resistance to CQ, is as a major threat. New treatment guidelines based on short and highly effective drug regimens as well as regional and Plasmodium genus-wide integrated strategies for the containment of antimalarial drug resistance in the Greater Mekong Sub-region need to be urgently developed.

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CHAPTER V

P. vivax morbidity after radical cure: a two-year cohort study in Central Vietnam

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Abstract

Introduction: Following successful malaria control activities over the last 20 years and the country's commitment to malaria elimination by 2030, the public health importance of *P. vivax* in Vietnam has recently increased. We investigated *P. vivax* morbidity after radical cure treatment in a 2-year prospective cohort study to determine the risk of recurrent infections and related risk factors.

Methods: The study was conducted between April 2009 and December 2011 in 4 neighbouring villages in a remote forested area of Quang Nam province, Central Vietnam. *P. vivax* infected patients were treated with chloroquine and primaquine and followed up monthly for 2 years. At each visit, malaria symptoms and a blood sample for malaria diagnosis by microscopy and molecular methods were collected. Time to first vivax recurrence was estimated by Kaplan Meir (KM) survival analysis, and risk factors for the first and subsequent recurrences identified by Cox regression. Spatial analysis of vivax recurrences during the first year of follow-up was also done.

Results: Among the 260 *P. vivax* patients recruited 240 completed the 10day radical treatment (0.5mg/kg/day) and 219 were followed for at least 12 months. Recurrent vivax infection were identified by molecular methods in 76.78% (171/223), by microscopy in 55.61% (124/223) of them. Only 37.67% (84/223) subjects had symptomatic recurrences. Median time to 1st recurrence by molecular methods was 118 days (IQR [59; 208]). The estimated KM cumulative risk of remaining free of recurrence by month 24 was 20.40% (95%CI [14.42; 27.13]) by molecular methods, 42.52% (95%CI [35.41; 49.44])) by microscopy, and 60.69% (95%CI [53.51; 67.11]) for symptomatic recurrences. The main risk factor for recurrence (first- or all-) was a prior *P. falciparum* infection in the previous 1-2 months. A space-time analysis showed significant clustering of recurrences (RR = 2.1, p<0.001) in Village 4 (Ca'dong ethnic group).

Conclusion: A high number of *P. vivax* recurrences, mainly sub-microscopic and asymptomatic, were observed after high-dose primaquine treatment. Prior *P. falciparum* infection was an important risk factor for vivax recurrences. Malaria elimination efforts need to address this largely undetected transmission of *P. vivax* to accelerate the 2030 elimination agenda.

Keywords: *Plasmodium vivax*, cohort study, chloroquine, primaquine, radical cure, recurrence, sub-microscopic infections, malaria elimination, Central Vietnam

Introduction

Radical cure of *Plasmodium vivax* malaria, the most prevalent species outside sub-Saharan Africa [1], remains a challenge as preventing subsequent relapses and onward transmission requires treatment of both circulating blood (schizonticidal drugs) and the dormant liver (hypnozoiticidal drug) stages [2]. Chloroquine (CQ) and primaquine (PQ) have been combined for this purpose since the late 1950s, and PQ -an 8-aminoquinoline licensed in 1952- remains the only anti-relapse therapy currently available on the market. Its use implies several important challenges, including the risk of acute hemolysis in Glucose-6-Phosphate-dehydrogrenase (G6PD) deficient patients and poor adherence to the 14-day treatment schedule. Moreover, the rapid spread of P. vivax CQ resistance will require alternative blood schizonticides and thus new combinations for radical cure [3]. Over the past sixty years, various PQ regimen have been used [4]; the World Health Organization (WHO) guidelines recommend PQ at 0.25 mg/kg/day for 14 days together with CQ or an artemisinin combination therapy (ACT) [5]. In South East Asia and Oceania, where the vivax Chesson strain is prevalent, a higher PQ daily dose (0.5 mg/kg/day; 7.0 mg/kg total dose) is recommended.

In Vietnam, the public health importance of *P. vivax* malaria has recently increased because of successful control efforts against *P. falciparum* [6], and

the recent country's commitment to malaria elimination by 2030 [7, 8]. Malaria transmission remains confined in forested areas of Central Vietnam, with the highest incidence along the international borders with Laos and Cambodia [9]. Between 2011 and 2015, while the annual incidence of clinical malaria dropped by 43.83%, and malaria deaths by 78.57% (3 deaths recorded in 2015), the relative frequency of vivax clinical malaria as compared to falciparum increased from 39.19% to 53.63%. During the same period, the national guidelines for vivax radical cure malaria were revised three times, the current recommendations being PQ at 0.25mg/kg/day for 14 days together with CQ for 3 days. However, treatment adherence is probably very low, mainly because health staff is reluctant to prescribe PQ in remote areas where treatment supervision is extremely difficult [10]. Between 2007 and 2009, in an attempt to comply with the WHO recommendations for South East Asia countries as well as to improve treatment adherence, Vietnam implemented a 10-day course of PQ at a daily dose of 0.50mg/kg [11]. In order to evaluate this new regimen, we conducted a cohort study in Central of Vietnam in which P. vivax patients received supervised treatment and were followed up monthly for two years to assess the risk of vivax recurrence and related risk factors.

Methods

Study site and population

The study was conducted between April 2009 and December 2011 in four neighboring villages (Villages 1-3 in Tra Leng- and Village 4 in the Tra Don commune) in the mountainous and forested district of Nam Tra My in Quang Nam province, Central Vietnam. Villages were organized in several scattered clusters of about 4 to 45 households each (total population =1,810 individuals according to the March 2009 census). A detailed description of the sociodemographic characteristics and malariometric indices of this study area has been published elsewhere [12]. Briefly, the study population belonged to the M'nong (Villages 1-3) and Ca Dong (Village 4) ethnic groups, living mainly from slash and burn agriculture and cinnamon plantations in forest fields. Malaria transmission is perennial with two peaks, May-June and October-November, and two main vectors species: Anopheles dirus sensu stricto and An. minimus sensu stricto [13, 14]. Malaria prevalence as determined by a baseline cross-sectional survey carried out in April 2009 was 7.8% by light microscopy (LM) and 23.6% by PCR, with 60% P. falciparum and the remaining *P. vivax* infections. The G6PD genetic polymorphism (Vianchiang mutation) was estimated below 1.5% in both males and females, with no difference between ethnic groups [12]. The study team was based at the Commune Health Center (CHC) in Tra Leng (Village 3) and was supported by a network of trained hamlet health workers for the cohort follow-up.

Data collection

P. vivax infected patients from the four study villages were first identified by malaria screening at baseline [12] and then by the passive case detection (PCD) at the CHC until the target sample size (see below) was reached. Inclusion criteria were: age \geq 3 years and \leq 60 years old, axillary temperature \geq 37.5°C and/or history of fever in the previous 48 hours, microscopically confirmed P. vivax mono-infection (asexual stage), permanent residency in the study area, ability and willingness to participate in the study confirmed by a written informed consent (from parents/guardians of minors<18 years old). Patients were excluded if they had any danger sign or severe malaria, concurrent acute or chronic condition (malnutrition, etc...), known allergy or intolerance to study drugs, PQ treatment within the past month, or if they were pregnant or breastfeeding. Study subjects were treated daily with CQ (25mg/kg over 3 days) and PQ (0.5mg/kg/day for 10 days) according the national guidelines [11]. During the first 10 days of follow-up, patients were examined daily and treatment intake was directly observed. Patients were then asked to attend the CHC at day 14, 21, and 28 or if they felt ill between scheduled visits. Treatment outcomes at day 28 were defined according to the standard WHO guidelines [15] and were published elsewhere [16]. From day 28 onwards, study subjects were visited monthly at home by the study team for the following 23 months. Between scheduled visits, patients if unwell were advised to consult their HHW or the study team at the CHC. At each visit (scheduled and non-scheduled), they were systematically interviewed, body temperature collected, and had a finger prick blood sample taken for LM (thick and thin film) and later molecular analysis (filter paper blood sample=FPBS). Any LM confirmed *P. vivax* infection identified during the monthly follow-up was treated with a 3-day course of CQ; PQ re-treatment was administered at the end of the monthly follow-up. In addition, confirmed *P. falciparum* infections (either mono- or mixed infections) were treated with a 3-day course dihydroartemisinin-piperaquine (DHA-PPQ) as per national guidelines.

Laboratory procedures

Malaria parasites species and density were identified by LM. Thin films were fixed with methanol for 15 - 30 seconds; both thin and thick smears were stained with 3% Giemsa solution for 45 minutes. *P. vivax* asexual stages were counted against 200 white blood cells (WBCs), assuming a mean WBCs

count of 8,000/µl. Quality control was done at the National Institute of Malariology, Parasitology and Entomology (NIMPE) in Hanoi by a senior technician blinded to patients' details. All positive blood slides and 10% of randomly chosen negative slides were double checked, and in case of discrepancy slides was read by a third senior technician.

FPBSs were first stored at 4°C until transferred to NIMPE where they were stored at -20°C until processing. DNA extraction was done using the QIAamp DNA Micro Kit (Qiagen, Hilden Germany), and a species specific semi-nested multiplex Polymerase Chain Reaction (SnM-PCR) to detect the four human *Plasmodium* species was performed as described elsewhere [17]. Quality control was done at ITM by a senior technician who blindly reanalyzed 10% of randomly chosen blood samples.

Data analysis

Between 2003 and 2007, late parasitological failure to CQ in different sentinel sites of Central Vietnam was between 0% and 5.7% [18]. Assuming 5% treatment failure and 10% loss to follow up during the first 28 days, a sample size of 205 patients would have 3% precision at 5% significance level ("CSample" command/EpiInfo6). Assuming a similar failure rate for PQ and 20% loss to follow up over the 2-year period, 231 patients would provide

similar precision; an additional 8% security margin was added and a total of 250 patients were included in the cohort.

Data were double entered and checked in Epidata version 3.1 (The EpiData Association, Odense M, Denmark), and analyzed using STATA version 11 (Stata Corp, College Station, TX). A vivax recurrence was defined as an infection detected after the initial post-treatment parasite clearance. The main study outcomes were the incidence of *P. vivax* recurrences defined as follows: *i*) all recurrences identified by PCR (="all PCR- detected recurrences"); *ii*) all recurrences identified by PCR and positive by LM (="patent recurrences"); and *iii*) all patent recurrences with malaria symptoms (body temperature \geq 37.5°C and/or other symptoms; ="symptomatic recurrences"). Any patent or symptomatic vivax recurrence detected during the first 28-day follow-up was considered as a treatment failure, the patient received rescue treatment (DHA-PPQ) and his/her follow-up was stopped [16]. LMconfirmed vivax recurrences detected at monthly visits, after the initial 28day follow up, were systematically treated by CQ and considered as independent events. However, sub-patent microscopic (SM) recurrences (detected only by PCR) were not treated and could be detected for several consecutive months. Therefore, they were not considered as independent events but rather as "positive PCR person-month" for each month with one or more SM infection. Summary statistics were used to describe the baseline characteristics of the cohort at inclusion and at subsequent visits. The study design was taken into account using the survey ("svy") command in STATA, with household as primary sampling unit and village as strata (alpha = 0.05).

Patients were censored at the time of the last visit, which corresponded to any of the following: *i*) completion of the 2-year follow-up; *ii*) loss to follow-up; iii) recurrence during first 28 days treated by DHA-PPQ and PQ; or *iv*) official end of study in December 2011 (one year after the last patient was included). The follow-up time was estimated for each participant from the day of recruitment (D0) until final censoring. If study subjects had been absent for at least two consecutive monthly visits, they were excluded from the analysis; if they missed one monthly visit but remained within the study site, their follow-up time was counted as person-time at risk without gap.

For LM-detected vivax recurrences (regardless of symptoms), individual cumulative 30 days at risk were generated for each monthly visit and adjusted for the prophylactic effect of intermittent antimalarial treatments, *i.e.* patients were considered not at risk of *P. vivax* recurrence during the 14 days after the start of CQ. The same was applied for any *P. falciparum* detected by LM and treated with DHA-PPQ. For events detected by PCR only, individuals were

always considered at risk of vivax recurrence as PCR could not distinguish between sexual and asexual parasite stages and therefore could be positive any day (outside the 14-day post-treatment prophylactic period).

Time to first *P. vivax* recurrence after treatment was analyzed by Kaplan-Meier survival analysis, which estimated the probability for patients to remain free of any vivax recurrence (PCR-detected, patent, or symptomatic).

The following variables were considered in the risk factor analysis for Pv recurrence: village, ethnic groups, sex, age, age groups, education level, occupation, bed net in house, economic level, year of entry, season of entry, parasite density at day 0, and "prior *P. falciparum* infection within 2 months before *P. vivax* recurrence" (including all *P. falciparum* infections identified either by PCR only (SM *P.f* infections) or by PCR and LM (patent *P.f* infections). In addition, we also explored if being treated with an antimalarial (ACT or CQ) since the previous visit before *P. vivax* recurrence was associated with a higher risk of *P. vivax* recurrence at the following monthly visit. Among risk factors, socio-economic level was computed using three different variables for livestock ownership, *i.e.* number of i) buffaloes, ii) cows, and iii) pigs, and by using principal component analysis as described previously [12]. The risk factor analysis was conducted both for the first- and

for all P. vivax recurrences. A uni- and multivariable adjusted Cox Proportional Hazard (PH) regression model (Hazard Ratio, HR) was used to determine significant risk factors for a first PCR-detected P. vivax recurrence (=first recurrence per subject). A Cox Conditional Gap Time (CGT) model was used for assessing the risk for multiple recurrences per subject over time [19]. In this model, each recurrence was analyzed separately and stratified by recurrence order, the underlying assumption being that a subject was not at risk of a second recurrence until the first recurrence had occurred. Thus, the conditional risk set at time for recurrence k (t_k) was made up for all subjects under observation at time they had had recurrence k-1. This method takes into account the total time at risk and the total number of recurrences analyzed. The "stcox" command in the CGT model accounted for the extra correlation due to repeated observations within patients by using the vce (cluster ID) option. If a patient was identified with a patent P. vivax infection during an unscheduled visit, i.e. between two monthly scheduled visits, this result was recorded as the only one for that specific month. If the result of unscheduled visit was negative, the result of scheduled visit was accounted for that month. The incidence rate was calculated by case/person-year and time to recurrence analysis with day at risk unit. The proportional hazard (PH) assumption was tested for each Cox model. Since the latter was not verified when including
all patients, especially with the variable year of recruitment, the data were analyzed separately for patients recruited in 2009 and those recruited in 2010.

The sign rank test was used to compare the median time interval to first recurrence with the median time intervals between consecutive recurrences identified by PCR, *e.g.* median time to 1^{st} recurrence *versus* median time between 1^{st} and 2^{nd} recurrence among patients with at least two recurrences, etc.

The QGIS software QGIS v.2.16 (QGIS developer team, Open Source Geospatial Foundation) was used to map all households of study subjects and to classify them according to the number of positive PCR person-months during their first 12 months of follow-up after enrolment. The SaTScan software v.9.3 (M Kulldorff and Information Management Services Inc, USA) was used to identify spatial clustering of households with positive PCR person-months considering the following characteristics: pure spatial analysis, Poisson probability model, latitude/longitude coordinates, report of most likely clusters with no geographical overlap of secondary clusters, maximum spatial cluster size equal to 50% of total population. The analysis was first done without adjustment for covariates, and then by including age and gender as covariates. SaTScan applied multiple circular windows across

the study area, each circle representing a possible cluster. Clusters were assessed based on 999 Monte Carlo simulations to determine the probability of observed frequency of *P. vivax* positive PCR person-months relative to expected frequency under the null hypothesis of no clustering. The null hypothesis was rejected if resulting p-values of assessed clusters were below 0.05 and the window with the maximum log likelihood ratio (LLR) was identified as the most likely cluster. The relative risk (RR) reported for each identified cluster was the estimated risk within the cluster divided by the estimated risk outside the cluster.

Ethical clearance

Ethical clearance was obtained from both the Ethical committee of the NIMPE, Hanoi, and of the University of Antwerp. The fundamental principles of ethics in research on human participants were upheld throughout the project. The study objectives and methods were first explained to the community leaders for their approval. Each study participant gave a written informed consent after being explained the study objectives and follow-up procedures as well as their right to withdraw without prejudice for themselves or their families. Parents/guardians of patients aged less than 18years old

signed the individual informed consent while those aged 12-18 were asked to provide their individual assent.

Results

Cohort characteristics

A total 260 *P. vivax* infected patients were identified and enrolled in the 2year follow-up between April 2009 and December 2010.



*LPF: Late Parasitological Failure **LCF: Late Clinical Failure PCR: Polymerase Chain Reaction LM: Light microscopy



Among them, 20 (7.69%) withdrew consent and did not complete the 10-day radical cure (Figure 1). By day 28 (=first monthly visit), 9 additional patients had withdrawn consent and 8 had treatment failure (treated with DHA-PQ and 10-day PQ) and were excluded. Therefore, the cohort included 223 patients after day 28; 4 additional patients withdrew consent during the following 23 months; 219 (98.21%) and 107 (48.86%) patients completed the 12-month and 24-month follow up, respectively. Total individual follow-up times ranged from 9 to 719 days, with a median of 628 days (IQR [508; 718]).

Most patients were males (sex ratio M: F=1.6), and children 3-9 years old represented almost half (43.08%) of the cohort (Table 1). The M'nong ethnic group (78.46%) in Villages 1-3 was the most represented. All adults were farmers. Most patients were very poor (lowest income category=56.54%) and without a bed net at home (70.77% at the start of the study). Beside fever and/or history of fever, the most frequent symptoms at enrolment were headache and fatigue. The spleen rate was 6.15% (16/260). The mean parasite density at enrolment was 2,754.1/µl and gametocytes were found in most infections (86.15%). By day 3, all patients had cleared infection and were symptom-free [16].

Variables	n	%	95%CI
Village			
Village 1	101	38.85	[34.83; 43.02]
Village 2	64	24.62	[21.25; 28.33]
Village 3	39	15.00	[12.84; 17.45]
Village 4	56	21.54	[17.63; 26.04]
Gender			
Male	159	61.15	[55.76; 66.82]
Ethnic groups			
M'nong	204	78.46	[73.96; 82.37]
Cadong	56	21.54	[17.63; 26.04]
Age groups			
3 9	112	43.08	[37.45; 48.89]
1019	71	27.31	[21.84; 33.55]
2029	44	16.92	[12.75; 22.11]
3060	33	12.69	[9.35; 17.00]
Occupation			
No (children < 6 years old)	70	26.92	[22.02; 32.47]
Farmer	85	32.69	[27.51; 38.34]
Pupil	105	40.38	[34.34; 46.74]
Bednet in house			
No	184	70.77	[61.81; 78.37]
Atleast one	76	29.23	[21.63; 38.19]
Economic status ^o			
Lowest income	147	56.54	[47.20; 65.43]
Low	26	10.00	[5.70; 16.96]
Higher	87	33.46	[25.18; 42.90]
Year of recruitment			
2009	126	48.46	[41.80; 55.18]
2010	134	51.54	[44.82; 58.20]
Parasite density at day 0 (per μl)			
≤ 1000	56	21.54	[16.90; 27.03]
1001 - 5000	104	40	[33.61; 46.75]
> 5000	100	38.46	[31.86; 45.52]
Asexual parasites/µl (geometric mean)	2,7	54.07	[2,271.87;
Infections with gametocytes	224	86.15	[81.37; 89.86]

Table 1. Baseline characteristics of the 260 patients enrolled in the cohort

Clinical symptoms											
Fever (axillary temperature \geq 37.5)	154	59.23	[53.06; 65.12]								
Headache	94	36.15	[30.42; 42.32]								
Fatigue	86	33.08	[27.23; 39.50]								
Dizziness	28	10.77	[7.51; 15.22]								
Enlarged spleen	16	6.15	[3.58; 10.39]								
No symptoms	66	25.38	[20.36; 31.16]								

°The score into tertiles defined as "high", "medium", and "low" economic status

Characteristics of vivax recurrences

During the 2-year follow-up, 171 subjects (171/223=76.68%) had at least one PCR-detected recurrence (median of 4 recurrences per patient (IQR [2; 6])), 124 (55.61%) at least one patent recurrence (median = 2/patient; IQR [1; 4]) and 84 (37.67%) at least one symptomatic recurrence (median = 1; IQR [1; 2]) (Table 2). The median time to first recurrence was 118 days (IQR [59; 208]) by PCR, 141 days (IQR [86; 237]) by LM, and 181 days (IQR [97; 316]) for symptomatic recurrence. Median time to first recurrence tended to be shorter (88 days (IQR [58; 174])) among patients with multiple PCR recurrences compared to those with a single PCR recurrence (data not shown).

Among the 4,604 visits done, 71 (1.54%) were unscheduled because of symptomatic vivax recurrences. There were 748 PCR-detected *P. vivax* recurrences ("positive PCR patient-months"), and 337 (45%) of them were

	All PCR-de	tected	Patent-	<u>.</u>	Symptomatic-		
<i>P.vivax</i> recurrences	n	%	n	%	n	%	
Patients free of recurrence	52	23.32	99	44.39	139	62.33	
Patients with recurrence	171	76.68	124	55.61	84	37.67	
1 recurrence only	36	21.05	43	34.68	53	63.10	
≥ 2 recurrences	135	78.95	81	65.32	31	36.90	
Number of recurrences/individual (Median [IQR]; max)	4 [2; 6]; 13		2 [1; 4]; 10		1 [1; 2]; 7		
Median time from enrolment to first recurrence (days), [IQR]	118 [59; 208]		141 [86; 237]		181 [97; 316]		
Time intervals from enrolment to first recurrence:							
by day 28	5	2.93	0	0	0	0	
29 - 180 days	113	66.08	80	64.52	42	50.00	
> 180 days	53	30.99	44	35.48	42	50.00	

 Table 2. Characteristic of P. vivax recurrences according to outcome definition: all PCR detected, patent and symptomatic recurrences

IQR: Inter Quartile Range

patent and included 139 symptomatic infections. The incidence rate was 1.98 per person-years (95%CI [1.84; 2.12]) for PCR-detected-, 0.89 per person-years (95%CI [0.80; 0.99]) for patent-, and 0.37 per person-years (95%CI [0.31; 0.43]) for symptomatic recurrences (Table 3). The majority of patients with PCR-detected (78.95%) and patent recurrences (65.32%) had multiple events while for symptomatic recurrences most patients (63.10%) experienced only one event.

A total of 285 *P. falciparum* infections were detected by PCR (median: 2 episodes/person (IQR [1; 3]), maximum 8/person); 240 (84.21%) of them were patent (median: 1 episode/person (IQR [1; 3]; max=8)) and 149 (52.28) symptomatic (median=1/person (IQR [1;2]; max=6)).

The cumulative risk of remaining free of recurrences was 20.40% (95%CI [14.42; 27.13]) by PCR, 42.52%; (95%CI [35.41; 49.44]) by LM, and 60.69% (95%CI [53.51; 67.11]) for symptomatic recurrences (Figure 2A). For *P. falciparum* recurrences, such cumulative risk by PCR was 55.61% (95%CI [48.79; 61.90]). Most of the first events for the three types of recurrences occurred during the first year, with little changes in the cumulative risk afterwards. The median survival time for PCR-detected

Dist factor			Univa	first recu	rrence	Univariate of all recurrences				
	KISK TACLOF		n/per-year	IR	HR	95% CI	n/per-year	IR	HR	95% CI
	Overall		171/148.64	1.15		[0.99; 1.34]	748/378.38	1.98		[1.84; 2.12]
	Village									
	- Village 1		68/76.40	0.89	1		288/161.07	1.79	1	
	- Village 2		40/34.48	1.16	1.18	[0.74; 1.87]	144/82.54	1.74	0.97	[0.79; 1.20]
es	- Village 3		22/24.33	0.9	0.91	[0.56; 1.48]	74/53.33	1.39	0.83	[0.61; 1.11]
enc	- Village 4		41/13.43	3.05	3.02**	[2.09; 4.36]	242/81.43	2.97	1.27*	[1.07; 1.51]
urr	Ethnic									
reci	- M'nong		130/135.21	0.96	1		506/296.95	1.7	1	
ed 1	- Cadong		41/13.43	3.05	2.92**	[2.06; 4.14]	242/81.43	2.97	1.32**	[1.13; 1.53]
ect	Prior <i>P.f</i> infe	ction be	efore <i>P.v</i> recu	rrence						
det	- No		154/147.09	1.05	1		645/369.41	1.75	1	
CR	- Yes		17/1.55	10.94	6.82**	[5.13; 9.06]	103/8.96	11.49	4.90**	[4.19; 5.73]
ΙЬ	Year of recru	itment								
P		-2009	99/63.39	1.56	1		526/212.85	2.47	1	
		-2010	72/85.25	0.84	0.57**	[0.42; 0.79]	222/165.53	1.34	0.69**	[0.57; 0.82]
	Bednet in hou	ise								
	- No		130/95.02	1.37	1		579/272.22	2.13	1	
	- At least one		41/53.62	0.76	0.59*	[0.39; 0.88]	169/106.16	1.59	0.86	[0.72; 1.04]

Table 3. Univariable risk factor analysis using Cox Proportional Hazard Model for first events and Cox Conditional Gap Time Model for all events (n=223).

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	Prior ACT/CQ treat	ment before <i>F</i>							
	- No					653/368.66	1.77	1	
	- Yes					95/9.72	9.78	5.08**	[4.32; 5.96]
	P.v density/µl at day	0							
	- < 1000	37/31.42	1.18	1		204/86.50	2.36	1	
	-6000	62/55.71	1.11	0.93	[0.62; 1.40]	252/138.44	1.82	0.83*	[0.69; 0.99]
	- > 5000	72/61.52	1.17	0.97	[0.66; 1.43]	292/153.44	1.9	0.85	[0.71; 1.01]
	Economic status								
	- Very low income	99/83.90	1.18	1		385/216.37	1.78	1	
	- Low	16/15.00	1.07	0.9	[0.49; 1.64]	66/35.14	1.88	1.01	[0.80; 1.27]
	- Higher	56/49.75	1.13	0.95	[0.66; 1.36]	297/126.87	2.34	1.21*	[1.04; 1.40]
	Overall	124/213.09	0.58		[0.49; 0.69]	337/378.38	0.89		[0.80; 0.99]
	Village								
	- Village 1	52/96.47	0.54	1		124/161.07	0.77	1	
ces	- Village 2	24/54.35	0.44	0.79	[0.45; 1.41]	53/82.54	0.64	0.82	[0.56; 1.20]
ren	- Village 3	11/37.85	0.29	0.57	[0.31; 1.04]	31/53.33	0.58	0.84	[0.53; 1.34]
inu	- Village 4	37/24.42	1.52	2.53**	[1.68; 3.81]	129/81.43	1.58	1.59**	[1.23; 2.07]
rec	Ethnic								
ent	- M'nong	87/188.67	0.46	1		208/296.95	0.7	1	
Pat	- Cadong	37/24.42	1.52	2.95**	[2.00; 4.35]	129/81.43	1.58	1.72**	[1.36; 2.18]
	Prior <i>P.f</i> infection be	fore <i>P.v</i> recu	rrence						
	- No	107/210.42	0.51	1		280/369.41	0.76	1	
	- Yes	17/2.67	6.38	7.97**	[5.57; 11.40]	57/8.96	6.36	5.16**	[3.93; 6.77]

	Year of recru	uitment								
		-2009	82/97.36	0.84	1		244/212.85	1.15	1	
		-2010	42/115.73	0.36	0.43**	[0.28; 0.65]	93/165.53	0.56	0.59*	[0.42; 0.83]
	Bednet in ho	use								
	- No		96/142.67	0.67	1		271/272.22	1	1	
	- At least one		28/70.42	0.4	0.61*	[0.39; 0.96]	66/106.16	0.62	0.72*	[0.53; 0.98]
	Prior ACT/C	Q treat	ment before A	P.v recu	rrence					
	- No						290/368.66	0.79	1	
	- Yes						47/9.72	4.84	6.39**	[4.62; 8.83]
	Overall		84/227.14	0.31		[0.25; 0.28]	139/378.38	0.37		[0.31; 0.43]
	Village									
	- Village 1		37/118.33	0.31	1		51/161.07	0.32	1	
ces	- Village 2		11/69.79	0.16	0.48*	[0.24; 0.95]	14/82.54	0.17	0.53*	[0.29; 0.97]
ren	- Village 3		10/40.72	0.25	0.78	[0.40; 1.54]	17/53.33	0.32	1.02	[0.55; 1.88]
uri	- Village 4		26/43.30	0.6	2.02*	[1.11; 3.69]	57/81.43	0.7	2.09**	[1.38; 3.15]
ree	Ethnic									
atic	- M'nong		58/228.84	0.25	1		82/296.95	0.28	1	
om	- Cadong		26/43.30	0.6	2.53**	[1.45; 4.41]	57/81.43	0.7	2.36**	[1.62; 3.45]
npt	Prior <i>P.f</i> infe	ection be	fore <i>P.v</i> recu	rrence						
Syr	- No		70/268.07	0.26	1		114/369.41	0.31	1	
	- Yes		14/4.07	3.44	9.68**	[5.67; 16.53]	25/8.96	2.79	5.89**	[3.73; 9.30]
	Year of recru	uitment								
		-2009	62/132.00	0.47	1		104/212.85	0.49	1	

	-2010	22/140.14	0.16	0.30**	[0.18; 0.49]	35/165.53	0.21	0.42**	[0.25; 0.71]
Bednet in hou	ise								
- No		63/191.87	0.33	1		113/272.22	0.42	1	
- At least one		21/80.28	0.26	0.78	[0.47; 1.30]	26/106.16	0.24	0.63*	[0.44; 0.92]
Prior ACT/C	Q treat	ment before <i>l</i>	P.v recu	rrence					
- No						115/368.66	0.31	1	
- Yes						24/9.72	2.47	5.96**	[3.87; 9.18]
<i>P.v</i> density/µl	l at day	0							
- < 1000		18/60.06	0.3	1		40/86.50	0.46	1	
	-6000	28/105.31	0.27	0.83	[0.47; 1.48]	35/138.44	0.25	0.59*	[0.38; 0.93]
- > 5000		38/106.77	0.36	1.09	[0.63; 1.87]	64/153.44	0.42	0.88	[0.57; 1.35]
Years of educ	cation								
- 0 (children)		34/89.10	0.38	1		64/134.14	0.48	1	
	-6	26/117.94	0.22	0.58*	[0.37; 0.93]	38/151.47	0.25	0.58*	[0.37; 0.92]
- 6+		24/65.11	0.37	0.96	[0.62; 1.49]	37/92.78	0.4	0.86	[0.57; 1.29]

n/per-year: Number of case per Person-years; IR: Incidence Rate; HR: Hazard ratio; * P-value<0.05; ** P-value<0.001. The following variables were systematically analyzed in both first and all recurrences model: village, sex, ethnic groups, age, age groups, occupation, years of education, bednet in house, economic levels, year of entry, season of entry, parasites density on day 0, prior *P. f* infection before *P. v* recurrence. In addition the variable "prior ACT/CQ treatment before *P. v* recurrence" was also analysed in the all recurrences model.

Table 4 Multivariate adjusted risk factor analysis using Cox Proportional Hazard (PH) model for first events, andCox Conditional Gap Time (CGT) model for all (multiple) events.

	First recurrence		<u>2009 A</u>	djusted	2010 Adjusted			
		HR	P-value	95% CI	HR	P-value	95% CI	
	Effect of ethnicity according to p	Effect of ethnicity= non-significant						
	- No <i>P</i> . <i>f</i> infection	2.21	0.002	[1.33; 3.67]	No interaction			
R	- P. f infection	0.07	0.059	[0.003; 1.11]				
PC	Effect of prior P. f infection before	re <i>P</i> . v re	ecurrence	according to	Effect of prior	P. f before	e P. v	
	- M'nong	99.19	0.001	[6.16; 1597.55]	1			
	- Cadong	2.98	0.002	[1.48; 5.99]	5.33	0.012	[1.45; 19.62]	
	Prior P.f infection before P.v recu	irrence						
P	- No	1			1			
_	- Yes	6.51	< 0.001	[3.66; 11.56]	5.92	0.018	[1.36; 25.75]	
T	Prior <i>P.f</i> infection before <i>P.v</i> recu							
X	- No	1			_			
S	- Yes	7.83	< 0.001	[4.25; 14.44]				
	All recurrences		20	09		2010		
	Effect of malaria treatment account	ding to	prior <i>P.f</i> i	nfection before				
	- No <i>P.f</i> infection	6.35	< 0.001	[4.32; 9.33]	8.94	< 0.001	[5.07; 15.77]	
R	- P.f infection	0.72	0.118	[0.47; 1.09]	1.53	0.482	[0.47; 5.02]	
P(Effect of prior <i>P.f</i> infection befor	e P.v rec	currence a	ccording to				
	- No malaria treatment	5.89	< 0.001	[4.34; 7.98]	5.06	< 0.001	[2.57; 9.96]	
	- Malaria treatment	0.66	0.09	[0.41; 1.07]	0.87	0.800	[0.29; 2.63]	

	Effect of malaria treatment acco	ording to	prior <i>P.f</i> ii	nfection before			
	- No <i>P.f</i> infection	21.40	<0.001	[10.61; 43.18]			
	- Pf infection	0.77	0.365	[0.44; 1.35]			
	Effect of prior <i>P.f</i> infection before						
	- No malaria treatment	6.33	< 0.001	[4.21; 9.54]			
Z	- Malaria treatment	0.23	< 0.001	[0.10; 0.50]			
F	Prior <i>P.f</i> infection before <i>P.v</i> rec	urrence					
	- No				1		
	- Yes				1.80	0.07	[0.96; 3.37]
	Prior ACT/CQ treatment before						
	- No				1		
	- Yes				13.83	< 0.001	[3.85; 49.72]
	Effect of malaria treatment acco	ording to	prior <i>P.f</i> ii	nfection before			
	- No <i>P.f</i> infection	6.22	< 0.001	[2.80; 13.82]			
	- <i>P.f</i> infection	1.07	0.88	[0.45; 2.54]			
7	Effect of prior <i>P.f</i> infection befo	re <i>P.v</i> rec	urrence a	ccording to			
X	- No malaria treatment	5.65	< 0.001	[2.81; 11.35]			
Ø	- Malaria treatment	0.97	0.95	[0.38; 2.46]			
	Prior ACT/CQ treatment before	<i>P.v</i> recu	rrence				
	- No				1		
	- Yes				6.05	0.001	[2.17; 16.86]

HR: Hazard ratio; PCR: Polymerase Chaine Reaction; LM: Light Microscopy; SYM: Symptomatic infection

recurrence was 174 days while this was more than double (356 days) for patent recurrences.

Time between PCR-detected recurrences tended to decrease with increasing number of events (Figure 2B); median time between 1st and 2nd recurrence (median=78days; IQR [43; 120], and between 2nd and 3rd recurrence (median=60days; IQR [40; 120]) were significantly shorter than that to 1st recurrence [patients with at least 2 recurrences: (median=88 days; IQR [58; 174]) (p=0.024); patients with at least 3 recurrences: median=88 days; IQR [58; 148]) (p=0.002)]. From the 4th to the 9th recurrence, the median time intervals remained stable at around 60 days, and from the 10th to the 13th recurrence 30 days. A similar pattern was observed with patent recurrences, the median time intervals stabilizing around 76 days from the 3rd recurrence onwards.

Figure 3 shows a heatmap of all patients'recurrences by month of follow-up ordered by the timing of first recurrence. SM recurrences occurred frequently in almost all patients and persisted for 2-3 months up to 7 months without or before becoming patent or symptomatic. The frequency of symptomatic and patent recurrences decreased with time, with a large majority of SM recurrences detected during the last 6 months of follow u



Wilcoxon-rank p-values were computed. * Second time interval was compared to first among patients with at least two recurrences; ** Third time interval was compared to first among patients with at least three recurrences; *** Fourth interval compared to first among patients with at least four recurrences

Figure 2. A) Overall Kaplan-Meier survival probability of remaining free of *P. vivax* recurrences (either all PCR-identified; patent-, or symptomatic recurrences) and remaining free of *P. falciparum* infection (by PCR) by month 24 of follow-up (n=223); B) Time intervals between consecutive *P. vivax* recurrences identified by PCR (n=223 patients)



Figure 3. Heatmap of *P. vivax* recurrences by month of follow-up among the 171 cohort patients with at least one recurrence.

Risk factors for P. vivax recurrences

In the uni-variable analysis, village 4, Ca'dong ethnicity, no bed nets in the house, being recruited in 2009 (vs 2010) and having a PCR-detected P. falciparum infection 1-2 months prior to the P. vivax recurrence were statistically significant risk factors for the 1st vivax recurrence, regardless of the detection method (Table 3). The same risks factors were found for all P. vivax recurrences, with the additional strong effect (HR>5.0) of a prior antimalarial treatment (ACT or CQ) within 1-2 months before the recurrence. Since village and ethnicity were highly correlated and only Village 4 (*i.e.* Ca'dong ethnicity) had a significant effect, only ethnicity was kept in the final model. Only ethnicity, prior falciparum infection, and year of recruitment remained significantly associated to 1st PCR-detected recurrence by the multivariable adjusted Cox PH regression model (Table 4). In addition, since the Cox PH test was significant for all three outcomes (PCR-detected, patent and symptomatic) - reflecting differences in HR over time - the risk factor analysis was computed separately for patients recruited in 2009 and in 2010. Among patients recruited in 2009, the final model for 1st PCR-detected recurrence showed a significant interaction term (HR=0.03; p=0.016) between ethnicity and a prior falciparum infection. Ca'dong ethnicity (compared to M'nong) increased the hazard more than 2-fold but only among those who had no previous falciparum infection (HR=2.21; 95%CI [1.33; 3.67] p=0.002). Conversely, the effect of a previous falciparum infection was much stronger among M'nong (HR=99.19; 95%CI [6.16; 1,597.55] p=0.001) than among Ca'dong patients (HR=2.98; 95%CI [1.48; 5.99]; p=0.002). There was no interaction for the risk of 1st patent or symptomatic recurrence among those recruited in 2009, *i.e.* only a previous falciparum infection significantly increased the hazard of 1st recurrence (HR=6.51 and 7.83 for patent and symptomatic recurrences, respectively). Among patients recruited in 2010, there was no interaction in the final model; only a previous falciparum infection remained highly significant for the risk of both 1st PCR-detected and 1st patent recurrence (respectively, HR=5.33; 95%CI [1.45; 19.62] p=0.012 and HR=5.92; 95%CI [1.36; 25.75] p=0.018). In addition, the final model for the risk of 1st symptomatic recurrence did not converge, probably due to the small number of events.

The final Cox CGT model for the risk of all recurrences among patients recruited in 2009 showed a significant interaction term between a prior falciparum infection and antimalarial treatment for all three outcomes (all PCR-detected recurrences: HR=0.11; p<0.001; patent recurrences: HR=0.035; p<0.001; and symptomatic recurrences: HR=0.17; p=0.03). The effect of prior antimalarial treatment (*vs* no treatment) was very strong (HR:

6.35; p<0.001) but only among those without previous falciparum infection, and this was much higher for patent recurrences (HR=21.40; p<0.001). Conversely, the effect of previous falciparum infection was only significant among those who had not been treated for malaria during previous visits and this was very similar for all PCR-detected, patent and symptomatic recurrences, i.e. HR=5.89 (p<0.001), HR=6.33 (p<0.001), and HR=5.65 (<0.001), respectively. For patients recruited in 2010, the final Cox CGT model for the risk of all PCR-detected recurrences showed a similar interaction between prior antimalarial treatment and falciparum infection (HR=0.17; p=0.008). This did not apply for the risk of patent- or symptomatic recurrences, as only prior antimalarial treatment remained strongly associated with the risk of both patent (HR=13.83; p<0.001) and symptomatic recurrences (HR=6.05; p<0.001).

Space-time clustering of vivax recurrences

Figure 4 shows the spatial distribution of households of the four study villages and the location of the most likely cluster of households with *P. vivax* positive PCR person-months during the first 12 months of follow-up



Figure 4. Clustering of *P. vivax* recurrences identified by PCR using Spatial analysis StaTScan among 219 patients during their first 12 months of follow-up.

for all patients who completed at least 12 months (n=219). The spatial analysis confirmed that PCR-detected vivax infections were non-randomly distributed. Indeed, the most likely spatial cluster of positive PCR personmonths (RR = 2.1, p<0.001) covered almost entirely Village 4, including 41 enrolled individuals (24/129=18.70%) in 41 households. This cluster represented 32.6% (142/435) of all *P. vivax* positive PCR person-months during the first 12 months of follow-up. The cluster location and size did not change after adding age and gender as covariates in the spatial analysis.

Figure 5 shows the study timeline with corresponding climatic data (Figure 5A) as well as the monthly evolution of the proportion of study patients identified with vivax recurrences together with the monthly incidence risk of *P. falciparum* infections (Figure 5B) detected in the four study villages. The first mass distribution of long lasting insecticidal nets (LLINs) in the study area was implemented in September 2010 and was followed by a steady decrease in both the risk of *P. falciparum* infections and of *P. vivax* recurrences (Figure 5B). The monthly incidence risk of *P. falciparum* decreased to almost zero from February 2011 until the end of follow up; similarly, the risk of all PCR detected and patent vivax recurrences by December 2011steadily decreased to 7% and 1%, respectively.



Figure 5. A) Monthly climatic data in the study area; B) Monthly evolution of the cohort size with proportion of patients identified with *P. vivax* recurrence (all PCR-identified-, all patent-, and all symptomatic recurrences) and the monthly incidence risk of *P. falciparum* infections detected by ACD and PCD (LM) in study population.

Discussion

This large and comprehensive study on a cohort of *P. vivax* patients in the Greater Mekong Sub-region had an exceptionally low drop-out rate; only 13 out of the 240 patients treated with radical cure did not complete the 2-year follow up. Post-treatment vivax recurrences were extremely frequent, most of them asymptomatic and undetectable by standard microscopy. Such high burden was unexpected as patients received high-dose PQ for ten days under direct observation. Although this study was not specifically designed to measure PQ efficacy itself, the high incidence of vivax recurrences in this low transmission setting suggests a lower-than expected PQ efficacy, at least at the dosage employed. P. vivax resistance to PQ remains difficult to establish for several reasons, including the lack of long-term P. vivax in vitro culture, the difficulty to work with non-human primates [20], potential key confounders such as PQ dosing, patients' adherence, parasite tolerance to PQ (Chesson strain), chloroquine resistance and the risk of P. vivax re-infections [21]. Although patients were administered the recommended daily dose for the radical cure of the vivax Chesson strain (0.5mg/kg/day), the total PQ dose over the 10-day course employed was 5mg/kg while according to the WHO guidelines, it should be 7.0mg/kg (0.5mg/kg over 14 days) [22]. This is why, despite careful dosing according to the body weight and the direct treatment

observation, the total dose of PQ given with the 10-day regimen may have been insufficient for radical cure. Indeed, in a systematic review of clinical studies done on the Chesson strain between 1946 and 2004, Baird & Hoffman [21] concluded that 0.5mg/kg for 14 days was the recommended dose in nonpregnant subjects without G6PD deficiency. Moreover, early clinical studies demonstrated that the main determinant of therapeutic efficacy was the total PQ dose rather than the daily dosing or duration of treatment and this was initially defined at 6.0mg/kg [23]. Later, such recommendation was changed to 7.0mg/kg over 14 days to reduce the risk of hemolysis and improve gastrointestinal tolerability [22]. A recent systematic review reported that a total PQ dose \geq 5.0mg/kg had the lowest risk of recurrence (median=0%) (range 0-15%)) compared to very low-(≤2.5mg/kg) and low total dose (>2.5 and <5.0mg/kg) [4]. Patients in our cohort received 5mg/kg of PQ as total dose which could be defined as "high dose". However, it is impossible to draw any conclusion on whether the 2mg/kg difference with the recommended total dose of 7mg/kg would have further decreased the risk of recurrence. An earlier study on high dose PQ regimen (30mg/day) given over different length of time did not show any difference between the 9-, 11- or 14-day regimen in preventing early relapses [24]. However, these results should be taken with caution as the follow-up was only 28 days and the study was done in Thailand where *P. vivax* may be more susceptible to PQ.

There may be no difference in terms of safety, efficacy and tolerability between a PQ total dose of 7mg/kg given over 7 or 14 days as suggested by Krudsood et al [24] while a shorter regimen would improve patients' adherence, a well-known limiting factor of PQ effectiveness [10, 25]. This is currently evaluated in a multi-centre trial implemented also in Central Vietnam (IMPROV) [26]. Nevertheless, the deployment of a high-dose 7-day PQ regimen in Vietnam would require systematic and reliable G6PD testing prior to treatment, a procedure currently not included in the national guidelines. Three barriers to the roll-out of routine G6PD testing were identified by policy makers and healthcare providers in four vivax-endemic countries (Bangladesh, Cambodia, China, and Malaysia): a perceived low risk of drug-induced haemolysis; the perception that vivax malaria was benign and PQ treatment not considered a priority; and, the additional costs of routine testing [27]. Cost-effectiveness studies on routine G6PD testing are currently ongoing as part of the IMPROV study, and preliminary data from the Thai-Myanmar border suggest potential reduction in total health care costs [28]. Additionally, the recent availability of reliable G6PD rapid diagnostic tests [29] should improve the financial and operational accessibility for communities in remote areas such as those that participated to our cohort study.

A substantial proportion of vivax recurrences were identified only by molecular methods. Although a proportion of these sub-patent infections may be the same blood stage infection, their persistence for a relatively long period of time (up to 7 months) probably contributes maintaining vivax transmission, though the extent of such contribution is unknown. However, considering that sub-microscopic vivax infections with gametocytes can successfully infect mosquitoes [30, 31], any detectable vivax blood stage infection, regardless of parasite density or presence of gametocytes, should be considered as potentially infectious. In addition, vivax recurrences are probably even more frequent than observed in our study as outside a research setting PQ is either not prescribed (often in remote areas as treatment is difficult to monitor) or not supervised.

Sub-microscopic vivax infections probably represent about 67% of all PCRdetected vivax infections [32] with a negative relationship, as for *P. falciparum* [33], between prevalence of patent and submicroscopic vivax infections [32]. Indeed, during the last 6 months of follow up of our cohort, when malaria transmission was significantly reduced, PCR-detected vivax recurrences were significantly more frequent than patent or symptomatic infections. Therefore, the current size of the human reservoir of P. vivax infection is probably much larger than observed in our cohort, and in low endemic settings such as in our study site in Central Vietnam current diagnostic tools and detection methods (ACD and PCD based on LM/RDT) are unlikely to interrupt transmission. Indeed, according to our survival analysis, ACD would have had detected at best only about half of vivax recurrences (patent recurrences), and PCD less than one third (symptomatic recurrences). While quality LM and RDT seem to be adequate tools for case management, identifying all infected individuals to reduce the human reservoir of infection requires more sensitive diagnostic tools, deployable in remote and resource poor settings [32, 34]. Molecular test remain the gold standard for the detection of low density infections but are expensive, require highly qualified staff and are difficult to deploy in remote areas. Ultrasensitive RDTs (uRDT) may be able to interrupt transmission when used in mass screening and treatment in low transmission settings such as in SEA, and could be deployed and evaluated in Central Vietnam [35, 36]. Nevertheless, available uRDTs can detect only P. falciparum. Ideally, next generation uRDTs should combine P. falciparum and P. vivax antigens, or have a pan-human malaria antigen. Alternatively, multiplex field PCR assays (LAMP) [37] are now also available and could be used to target the human reservoir of infection in Vietnam and other SEA countries.

A substantial proportion of observed recurrences were probably relapses as malaria transmission in the study area was low at recruitment and further decreased during follow up. Assuming the risk of new infections is similar for P. vivax and P. falciparum, the difference in the KM survival curve of the cumulative risk of PCR-detected infections between P. vivax and P. falciparum should represent the relapses. According to this assumption, more than half of the first PCR-detected recurrent infections were relapses. This is probably an underestimation as the baseline survey in the study area carried out in April 2009 showed a higher risk of P. falciparum (92%) than P. vivax infections [12]. In PNG, it was possible to estimate that in a cohort of children treated with artesunate alone or combined with PQ (14-day at 0.5mg/kg/day) relapses contributed to approximately 50% of the observed recurrent P. vivax infections detected by PCR and that the effect of PQ was mainly observed during the first three months of follow-up [38]. When considering that the efficacy of the 14-day high dose PQ regimen on the Chesson strain prevalent in PNG could be 80% [39], the proportion of relapses could be even higher than 50%.

The frequency of submicroscopic infections may also cause chronic anaemia. Indeed, more than a third of our patients were anaemic (Hb<11.0g/dl) at baseline, before treatment, and showed substantial haematological recovery at day 28 post-treatment [16]. Such an effect may have been only transient given the high incidence and length of non-treated recurrent vivax infections. Unfortunately, Hb was not measured during the follow up and this is a limitation of this study. The relationship between anaemia and SM vivax infections is unclear though in Brazil such association was observed [40] and in PNG it was also reported for SM *P. falciparum* as well as asymptomatic infections with both species [41]. It is likely that the high incidence of PCR-detected vivax and falciparum infections (and co-infections) in our cohort would cause a substantial burden of chronic anaemia.

Prior *P. falciparum* infections, drug use, ethnicity or geographic location, and malaria transmission intensity were identified as risk factors for vivax recurrences. These are well-known and already described risk factors [42]. The effect of transmission intensity is suggested by the fact that the PH assumption could not be held when year of recruitment was kept in the model, indicating a significant change in HRs over time as both *P. falciparum* and *P. vivax* incidence decreased substantially between 2010 and 2011. Indeed, patients recruited in 2009 were significantly more exposed to *P. falciparum*

and *P. vivax* infections, particularly Ca'dong people in Village 4, explaining the significant interaction between ethnicity and prior *P. falciparum* infection for the risk of first recurrence in patients recruited in 2009 but not in 2010. Differences in the risk of infection could be due to different host- (human genetics, acquired immunity, socio-cultural) and parasite- (genetic diversity) related factors [32]. As the Ca'dong community resided only in Village 4, which was geographically isolated from the three other villages and the health center, it is not possible to estimate the relative contribution of each risk factor. A prior population genetics analysis of all vivax infections prior to treatment showed a significant parasite genetic differentiation and population structure between Village 4 and the other three villages, possibly explained by geographical barriers (river, remoteness) [43].

P. falciparum infections were identified as the main risk factor for vivax recurrence (PCR detected, patent-and symptomatic), regardless of the year of recruitment. The triggering effect of symptomatic *P. falciparum* infections on *P. vivax* relapses is known since the early observations in 1920s' by Synton India [44] and has been extensively reported in Asia [42, 45, 46]. In the multivariable analysis for multiple recurrences, there was a significant interaction between prior *P. falciparum* infections and prior antimalarial treatment (systematically administrated for patent malaria infections

regardless of symptoms), suggesting that falciparum infections were independently associated with recurrences only when patients did not received antimalarial therapy (either CQ or DHAPQ). For treated falciparum infections the effect was protective (although it did not reach statistical significance), possibly reflecting the prolonged prophylactic effect of DHAPQ [45]. Therefore, chronic submicroscopic *P. falciparum* infections can trigger vivax recurrences, contributing to increasing the human reservoir of *P. vivax* infections. Considering that about 50% of *P. falciparum* infections are submicroscopic [47], in areas of co-endemicity, the contribution of *P. falciparum* in maintaining *P. vivax* transmission may be substantial; and decreasing *P. falciparum* prevalence may thus affect also *P. vivax* transmission.

The pattern of recurrences, *i.e.* early and frequent recurrences, observed in our cohort is typical of the parasite strains described in South East Asia [42, 48]. Although the median time to recurrences varied by their number, some patients experienced only one recurrence with a significantly longer median time (168 days) which may indicate a different strain with a different relapsing pattern. An *ad-hoc* analysis for the risk of having only one- *vs* multiple recurrences did not identify any significant risk factor (results not shown).

Our study had some limitations, including the lack of control arm which could have allowed the estimation of the relative contribution of new infections and relapses [38] but for ethical reason this option was not chosen. Instead, priority was given to the comprehensive assessment of post treatment morbidity with the newly implemented radical cure regimen in Vietnam. Hb should have been monitored throughout the follow-up in order to determine the risk and severity of anemia related to vivax malaria. Finally, in our analysis, the different sampling schemes underlying scheduled and unscheduled visits was not considered. Nevertheless, the related bias is probably minimal because of the intense monthly sampling and the moderate number of unscheduled visits (4.533 scheduled visits versus 71 unscheduled visits). Further research should focus on developing appropriate methodology take into account the outcome-dependent sampling underlying to unscheduled events, especially in case of larger time gaps between scheduled visits [49].

In conclusion, this comprehensive cohort study identified a high burden of post-treatment vivax infections, mainly asymptomatic and sub-microscopic, despite a high-dose PQ regimen (total 5mg/kg) administered under direct observation. The main risk factor for vivax recurrence were prior *P*. *falciparum* infections, particularly the submicroscopic ones which were

untreated and persisted for several months, and prior CQ mono-therapy indicating vivax drug resistance. When considering Vietnam reverted to the standard PQ dose of 0.25mg/kg (total dose 3.5mg/kg), which is often not prescribed or supervised, the actual parasite reservoir for both *P. falciparum* and *P. vivax* [12] is likely to be much larger than observed in our cohort. This is enhanced by the inability of the current surveillance methods based on LM or RDT to detect a substantial proportion of infections. This, besides the consequences on individual health (chronic vivax-related morbidity), represents a major problem for reaching the goal of malaria elimination by the year 2030 endorsed by the Vietnamese government. Therefore, field trials are urgently needed to assess the efficacy and cost-effectiveness of novel treatment approaches tackling simultaneously *P. falciparum* and *P. vivax* infections, together with ultra-sensitive and field deployable rapid diagnostic tests for both *P. falciparum* and *P. vivax*.

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CHAPTER VI

Discussion and Conclusions

Discussion

Our research included two types of studies, *i.e.* a baseline cross-sectional survey followed by a 2-year prospective cohort study, in an attempt to characterize malaria epidemiology in a typical population at risk of Central Vietnam, *i.e.* impoverished ethnic minority living in a remote forested area. In both studies, we used LM and PCR (SnM-PCR) [1, 2] in order to identify either the prevalence or incidence of malaria infections. We found a high prevalence of asymptomatic and sub-microscopic with P. falciparum and P. vivax malaria infections (Chapter 3). Moreover, our results allowed to describe, for the first time in Vietnam, the resistance of *P. vivax* to CQ (Chapter 4). Finally, the two-year monthly follow-up of 240 P. vivax infected individuals showed a high risk of P. vivax recurrences, mainly asymptomatic and sub-microscopic infections, despite a radical cure treatment (CQ+PQ) with a total PQ dose of 5.0mg/kg directly supervised for 10 days (Chapter 5). In addition, prior *P. falciparum* infection was the main risk factor for all three outcomes (i.e, all PCR-detected-, all patent-, or symptomatic recurrence) and for both the first and all vivax recurrences.

Impact of research outcomes to Vietnam National Malaria Control Programe

Current strategies of the National Malaria Control Programe (NMCP) in Vietnam are based on control phase in zone 4, 5 (moderate and high malaria transmission) and elimination phase in zone 2, 3 (resurgent malaria and low malaria transmission) following malaria stratification results in 2014. Malaria cases detection is based on PCD and ACD activities with PCD applied in both phases, while ACD is changing to reactive active case detection (re-ACD) in elimination phase. Indeed, re-ACD is carried out following introduce case which is confirmed by cases investigation and classification. Currently, in both PCD and ACD activities, the diagnostic tools including RDT/LM are using to detect malaria infection. In our study area with high malaria transmission, the ACD was carried out and it recorded the high number of sub-microscopy which challenge to current malaria elimination effort. To overcome this issue, molecular diagnosis and high sensitive RDT should be used in routine ACD and PCD [3].

Chloroquine resistance: Regarding to minimum inhibitory of chloroquine (MIC) blood concentration, our study confirmed *P. vivax* resistance chloroquine after 28 days of following *P. vivax* patients (Chapter 3). However, the new Vietnam national guideline of treatment malaria in 2016 given 3 days of CQ (10 mg/kg/day of CQ for two days and 5 mg/kg/day in the third day) + 14 days of PQ (0.25 mg/kg/day) treatment *P. vivax* infection [4]. Our finding of PvCQR is only one evidence *P. vivax* resistance chloroquine in Vietnam while primary analysis of 42 days efficacy study of CQ alone treatment in Gia Lai province in 2015-2016 reported high efficacy of CQ and no evidence of cloroquine resistance in the area (data of NIMPE not yet publication).

Overall, our finding recommended to NMCP using high sensitivities of RDT and diagnosis tools in routine PCD and ACD to detect sub-microscopy and asymptomatic infection. Monitoring and efficacy of chloroquine treatment *P*. *vivax* infection will be carried out in difference malaria transmission setting

to collect more evidences of PvCQR to convince NMCP changing new regimen treatment *P. vivax* infection.

High proportion of sub-microscopy and asymptomatic infection

Our cross-sectional study done in Quang Nam province in April 2009 (towards the end of the dry season) found an overall malaria prevalence of 23.6% by PCR, which was about three-fold higher than by LM (7.8%), most of these infections being asymptomatic (75.4%). High proportions of asymptomatic infections in cross sectional surveys have been repeatedly reported since more than ten years from different Central provinces such as Binh Thuan (81%) [5], Ninh Thuan (~80%) [6], Quang Tri province in 2015 (98.1%) [7] as well as from other GMS countries [8-10]. More recently, with the availability of molecular diagnostic tools increasing number of reports from Central Vietnam indicate that the parasite reservoir is actually much larger (with a majority of sub-microscopic infections) and diverse than previously thought by LM [1, 5]. Indeed, out of the 29.1% (671/2,305) Plasmodium-positive samples by PCR, only 14.4% (331/2,305) were detected by LM [6].

Currently, the detection of sub-microscopic infections is a challenge to ongoing elimination efforts, as the most commonly used diagnostics tools in the country continue to be LM and RDT. LM is the "gold standard" for malaria parasite detection in Vietnam, but the quality of the microscopic readings varies substantially across levels of the health system as shown by the regular quality control studies done by NIMPE [11, 12]. Indeed, most of the high quality LM diagnosis (skilled staff) concentrate in central laboratories such as district, provincial and regional level, while most of the

malaria infections occur in poor and remote rural communities [11, 12], where microscopy reading capacities are often weak. To overcome the poor quality of LM in remote areas, RDTs represent a useful diagnosis tool for malaria case management. Since 2005, the NMCP has used different types 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 *P. falciparum/P. vivax* (Standard diagnostic, INC, Korea) and ICT *P. falciparum/P. vivax* (ICT-Amrad, Sydney, Australia) [13, 14]. Currently, NMCP is using SD Bioline Malaria Ag *P. falciparum/P. vivax*, which is supported by the Global Fund. However, given the long history in Vietnam of widely implemented LM with a comprehensive quality control system and systematic retraining of microscopists RDTs remain overall less sensitive than LM (*ie*,100 parasites/µl threshold *versus* 60 parasite/µl) [14] while they also miss other less prevalent species such as *P. malariae*, *P. ovale*, *and P. knowlesi* [13, 14].

To overcome the limitations of both LM and RDTs, PCR techniques have been extensively used for diagnosis and quality control due to their high sensitivity (1-5 parasites/ μ l of blood) and specificity, particularly with low parasitemia and mixed infections [15-17]. A systematic review of studies using PCR detection methods (39 study sites in 22 countries) classified malaria transmission (both for *P. falciparum* and *P. vivax*) based on the proportion of asymptomatic infections detected by PCR and in all age groups: low- (0-16.8%), moderate- (9.6-58.9%) and high malaria transmission (16.3-28.5%) [18]. In low transmission settings, a review of 44 cross-sectional surveys showed that an average of 55.7% of *P. falciparum* and 69.5% of *P. vivax* infections were only detected by PCR [19]. Another large crosssectional survey in the Thailand-Myanmar border areas, Cambodia, and Vietnam recorded only 4% and 5% malaria prevalence respectively by RDT and by LM and 20% by real-time PCR [20]. A review of 106 different surveys estimated that asymptomatic and sub-microscopic infections account for 20-50% of all human-to-mosquito transmissions [3]. Our results suggest that the inclusion of molecular tools in re-ACD would substantially increase the detection rate and treatment of malaria infections, hence effectively target the parasite reservoir of infections which remains largely undetected. Currently, the use of standard PCR techniques for re-ACD is impractical because of their cost, infrastructure and training requirements, and long turn-around times. However, field PCR techniques such as loop-mediated isothermal amplification (LAMP) and real-time PCR methods have been developed [21] to detect malaria in the field working condition, while results are available within 2 hours [22-24]. A study carried out in Zanzibar, reported that the LAMP is a field-friendly, sensitive diagnostic test that could be useful for mass screening and treatment malaria campaigns which require quick results to enable prompt treatment [25]. To date, this method is under evaluation in Vietnam and if successful, LAMP will be used for re-ACD, which is likely to contribute substantially to reduce transmission assuming that most of the submicroscopic infections which are potentially infectious to mosquitoes.

In our survey (Chapter 3), approximately a third (36.3%) of all malaria infections were found to carry gametocytes by LM. This is probably a large underestimation compared to what PCR detection methods usually detect. Indeed, studies using RT-qPCR tools can detect *P. falciparum* gametocytes at a threshold of 0.3 gametocytes/µl blood [26], with estimated *P. falciparum* gametocytes prevalence 10-20 times higher than those detected by LM [27,

28]. A previous study in Kenya indicated that female Anopheles mosquitoes can be infected by P. falciparum gametocytes even at very low density (5 gametocytes/µl), even those undetectable by real-time quantitative nucleic acid sequence-based amplification (QT-NASBA) method [29]. A recent study in Colombia assessing P. vivax gametocytes density able to infect Anopheles mosquitoes showed that asymptomatic carriers, with presumably long-lasting infections, presented the highest proportion of mature gametocytes and were as infective as patients with acute clinical symptoms [30]. Overall, molecular tools are much more sensitive to detect malaria infections, identify species and sexual stages than the still routinely used RDT and LM. In addition, molecular tools can address additional questions concerning malaria transmission in elimination settings, such as the detections of sub-microscopic infections, species distribution, gametocyte prevalence and density, as well as to identify risk factors for gametocyte carriage in the population [3]. NIMPE is has implemented RT-qPCR assays for the detection and quantification of P. falciparum and P. vivax gametocytes in epidemiological studies.

P. vivax resistance to chloroquine

PvCQR *in vivo* is formally defined as the persistence of asexual *P. vivax* blood stages despite adequate whole blood or plasma levels of CQ (100 ng/ml in whole blood) [31]. By strictly applying this definition, PvCQR resistance has been confirmed largely through the malaria endemic world [32, 33]. Our results confirmed PvCQR for the first time in Vietnam with a treatment failure of 3.5% (Chapter 4). This was totally unexpected since *P. vivax* sensitivity to CQ was regularly monitored and considered sensitive as 100% ACPR was reported between 2006 and 2011 [34]. For the radical cure of *P. vivax*

infection, which includes the elimination of liver hypnozoites, PQ has to be added to the drug regimen at a dose of 0.5 mg/kg/day for 14 days (total dose 7.0mg/kg) in SEA countries following WHO recommendations [35].

The results of theraperutic efficacy study (TES) are the main basis for determination of the national treatment policy by the National Malaria Programme (NMP). The key outcome indicators of TES are the proportion of patients who are parasitaemic on day 3 and the proportion of patients with treatment failure by day 28 or day 42. To ensure the efficacy of the malaria treatment selected for national policy, WHO recommends a change in the national malaria treatment policy if the total treatment failure rate is $\geq 10\%$ (as assessed by TES) and that the NMP adopts antimalarial medicines with a parasitological cure rate of > 95%. In between 2015 and 2016, the study of CQ treatment alone among 59 P. vivax patients in Gia Lai province central part of Vietnam reported ACPR on D28 was 100% and ACPR on D42 was 79% and the preliminary analysis indicated no evidence of cloroquine resistance in the area (data of NIMPE not yet publication). Therefore, chloroquine remains the first-line treatment for P. vivax malaria in Vietnam and the NIMPE continues to closely monitor CQ efficacy in P. vivax infections [4]. Several studies found the delayed parasite clearance and recurrence were strongly correlated [36-39]. Moreover, a systematic review and meta-analysis of P. vivax drug trials showed that clearance of parasitaemia (by LM) in all patients by day 3 or in 95% of patients by day 2 was 100% predictive of CQ sensitivity in the study population, as defined by the day 28 recurrence rate. Therefore, further studies are needed to investigate early parasite clearance for monitoring of P. vivax in different endemic settings [40].

CQ resistance has been associated with single nucleotide polymorphisms (SNPs) in the *P. vivax* multidrug resistance gene 1 (*pvmdr1*) at codon (c) 976 (Y976F) [41] and it has been used in a recent study conducted in Gia Lai province central part of Vietnam. However, preliminary analysis of the association of these mutaitons with ex vivo phenotypes showed no indication of association (Dr. Nguyen Van Hong' NIMPE personal comunication). Similarly, a study of natural parasite populations of *P. vivax* from Papua, Indonesia, where PvCQR is highly prevalent, a sequence polymorphism in *pvmdr1*, translating into an Y976F substitution, was found in all patients presenting at a local health facility. The same substitution, however, was rare in Thailand, where PvCQR remains infrequent [42]. In a different study, another sequence polymorphism in *pvmdr1* (F1076L) was identified in *P. vivax* samples from Thailand and Indonesia [43]. Finally, several markers have been associated, but we still lack a strong marker with association in all studies. This continue to be one of the major lacks in the study of PvCQR.

Alternative treatment regimens in PvCQR are available and are currently based in ACTs (e.g. dihydroartemisinin-piperaquine), which have proven their efficacy against PvCQR [44-46]. Their use for *P vivax* infections presents many advantage in areas co-endemic for both *P. falciparum* and *P. vivax* [47]. In summary, in order to reach the goal of malaria elimination in Vietnam, the NMCP will be improving methods for monitoring of drug resistance especially in *P.vivax* species by combining the measurement of parasite clearance time and the prevalence of molecular makers following WHO guidelines. If in the near future, the prevalence of PvCQR is confirmed with treatment failure rate \geq 10%, the NMCP will need to envisage a change in treatment policy to ACT [48, 49] as already implemented in PNG.

Burden of P. vivax recurrences after radical treatment

In our longitudinal study, we used two types of regimens to treat P. vivax mono infections detected by LM. First, at enrollment, all patients were treated with a radical cure regimen (CQ+PQ) with 0.5mg/kg/d of PQ for 10 days under direct observation to clear both blood and liver stages. Patients were then monitored monthly to observe the time to first P. vivax recurrence as well as the rate of P. vivax recurrences during a follow-up period of up to two years. During the follow-up, all P. vivax recurrences detected by LM were treated with a CQ monotherapy to clear P. vivax blood stages (Chapter 5). This resulted in an overall high burden of vivax recurrences due to relapses, new infections and recrudescent infections. Half of the cohort patients had already presented a first vivax recurrence (by PCR) after about six months of follow up and by month 24, only 20% of patients remained free of vivax recurrence. When considering microscopy patent recurrences, the median survival time was about one year, while by month 24 less than half (42%) of the cohort patients were free of patent recurrence. Moreover, the median time to first recurrence was relatively short, *i.e* less than 3 and 4 months, respectively for all PCR and patent recurrences. This represents a high morbidity especially when considering full adherence (DOT) to the high dose PQ regimen, ie. 5mg/kg bw over 10 days. The current WHO recommendation is 7mg/kg bw over 14 days in South-East Asia and Oceania, where tropical strains with frequent-relapses are prevalent [35].

It is difficult to determine whether our regimen was under-dosed and what was the exact contribution of relapses and new infections, though the former are likely to represent the majority of recurrences as discussed in Chapter 5. Most worrying in terms of malaria elimination, the majority of these recurrent vivax infections would remain undetected with the currently applied surveillance strategy of PCD, and even re-ACD, with LM/RDT the later being implemented only in areas of artemisinin resistance. In addition, our study found high incidence of SM of *P. vivax* and *P. falciparum* infection which could contribute to chronic anaemia in this area (Chapter 5). Therefore, high sensitivity detectable tools of malaria parasite could also contribute to reduce the burden of chronic anaemia.

Overall, a total of 411 sub-microscopic (54.95% of all PCR detected recurrences) and 198 asymptomatic recurrences (58.75% of total LM detected). The prolonged follow-up with systematic monthly detection of malaria infections using PCR has enabled the identification of chronic submicroscopic vivax infections *(ie, on average 2-3 up to 7 months)*. This is an important finding as it challenges directly the current elimination efforts in Vietnam which are still based on standard diagnostic tools such LM and RDT. Therefore, our findings call for urgently including new highly sensitivity diagnostic tools deployable in remote field conditions such as field PCR or ultra-sensitive RDTs They are detected by PCR combining LM in both re-ACD and PCD. The sub-microscopic should be follow-up to become to patent or negative. Regarding to detect relapses of *P. vivax*, re-ACD should repeat regularly in area where *P. vivax* is major parasite or residual remaining.

Despite being unique in its abilities, PQ can produce acute haemolytic anemia in individuals who are glucose-6-phosphate-dehydrogenase (G6PD) deficient [50]. Therefore, it requires testing for G6PD deficiency before deciding to administration [51]. Although, tests for G6PD deficiency are available, more sensitive and cheaper point of care tests for G6PD deficiency are needed [52]. Currently in Vietnam, cost effectiveness of G6PD deficiency rapid test is studying in the field. A previous study in Vietnam showed that the prevalence of G6PD deficiency in the Kinh and the Mong ethnic groups is low (0.5% and 0.7%, respectively), but this study was performed in area without malaria transmission. In malaria transmission areas, the prevalence of G6PD deficiency among ethnic groups living in the foothills of 4 provinces in the North of Vietnam (Thanh Hoa, Son La, Ha Giang, and Hoa Binh) ranged from 9.7% to 31% [53]. However, there is no reported case of acute hemolysis cases due to G6PD deficiency in Central and Southern Vietnam so far.

The short half-life of PQ requires that it is administered over a long course of 14 days, which leads to poor compliance [54]. To overcome this issue, there are several alternative compounds that have been tested in various stages of clinical trials as a replacement for primaquine (tafenoquine, bulaquine, tinidazole and inidazolidinone) [55, 56]. In a recent multicentric study, the protective efficacy of tafenoquine was 92.6% (95% CI: 7.3%-99.9%; P=0.042) significantly greater compared to other regimens (CQ and CQ+PQ 15mg/day for 14 days) up to eight weeks of follow-up [57]. Probably the major potential advantage of tafenoquine over PQ is its ability to ensure a better compliance with therapy as the treatment course can be shorter.

In summary, radical cure regimen can prevent relapse of *P. vivax* in this area in order to monitor efficacy of PQ and CQ separately following to WHO's protocol. Testing G6PD deficiency are required to all patients before deciding to administration of PQ orothers in 8-aminoquinoline as tafenoquine in near future. Re-ACD should be regularly implemented with the use of molecular tools in combination to LM/RDT to detect asymptomatic and submicroscopic *P. vivax* for alternative treatment.

Molecular epidemiology

Molecular methods have been used in malaria epidemiology for almost two decades and theses tools allow 1) more sensitive estimations of prevalence and incidence that include sub-microscopic infections; 2) assessment of the effectiveness of intervention strategies on the occurrence, complexity, and duration of infections; 3) differentiation between recrudescent, relapsing, and new infections; 4) estimation of the effect of interventions on the allele frequency of the targeted gene (e.g., mutations associated with drug resistance or variants in a vaccine construct); 5) estimation of the differential contribution of individual hosts to transmission by targeting gametocyte-specific genes; and 6) assessment of demographic patterns within parasite populations (gene flow–migration–colonization of new areas and population expansions), especially when transmission is driven by specific groups of particularly mobile subclinically infected individuals or migration across borders [58].

Conclusions

After twenty years of continuous success in the NMCP, Vietnam officially engaged into malaria elimination in 2011 and set the goal of a malaria free country by 2030. Consequently, the NMCP shifted from control to elimination strategies, *ie* from reducing mortality and morbidity below public health importance to interrupting transmission by tracking and treating systematically all malaria infections irrespective of the presence of symptoms. The ACD is an important activity to contribute to the detection and treatment of malaria infections. re-ACD is being carried out in malaria elimination phase in low malaria transmission zone. However, this elimination phase is facing multiple challenges, mainly due to the fact that residual transmission occurs mainly in remote forested areas of Central Vietnam inhabited by very poor ethnic minorities where transmission is driven by an extremely powerful malaria vector which feeding and resting habits challenge standard vector control measures such as IRS and LLINs. Besides the urgent need to eliminate *P. falciparum* malaria to tackle the rapid spread of multi-resistant strains, the elimination of *P. vivax* malaria represents another major challenge due to biological charactersitics which make this species particularly difficult to control. Moreover, as in all areas of decreasing endemicity, the Vietnam NMCP is faced with the increasing importance of submicroscopic and asymptomatic infections, which are not detected by the current surveillance strategies based on ACD and standard diagnostic tools (LM/RDTs).

The research carried out in the framework of the present thesis confirmed and identified several important findings which have important implications for malaria elimination in Vietnam. First, high proportion of asymptomatic and submicroscopic infections is required more sensitivity detection tools should be applied to detect all malaria infection. PCR-based techniques are sensitive tools for detecting malaria infections with low parasite density. Thus, field PCR techniques such as the LAMP should 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. Second, our study result is the first confirmed evidence of *P. vivax* resistance to CQ in Central Vietnam while current treatment policy uses CQ as first-line of treatment *P. vivax* in Vietnam. Therefore, PvCQR should

continue to be monitored in different sentinel sites of central Vietnam, using standardized and sufficiently powered in vivo protocols of WHO with CQ monotherapy and with PQ therapy delayed to day 28. In addition, Vietnam has committed to malaria elimination by 2030, and within this con-text, antimalarial drug resistance, not only *P. falciparum* resistance to arteminins but also *P. vivax* resistance to CQ, is as a major threat. New treatment guidelines based on short and highly effective drug regimens need to be urgently developed.

To reach the target of malaria elimination, Vietnam malaria control programe requires more research to understand the contribution of sub-microscopic infections to malaria transmission, new antimalarial drugs, validate field PCR techniques. Beside of real-time report, malaria surveillance should be supported by molecular tools for accelerating malaria elimination within this country.

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Biography

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Publications

- Short Report: A Modified Semi-Nested Multiplex Malaria PCR (SnM-PCR) for the Identification of the Five Human Plasmodium Species Occurring in Southeast Asia
 Nguyen Van Hong, Peter van den Eede, Chantal Van Overmeir, Indra Vythilingham, Anna Rosanas-Urgell, Pham Vinh Thanh, Ngo Duc Thang, Nguyen Manh Hung, Le Xuan Hung, Umberto D'Alessandro, and Annette Erhart
 Am. J. Trop. Med. Hyg, 89(4), 2013, pp. 721–723, doi:10.4269/ajtmh.13-0027
- Epidemiology of forest malaria in Central Vietnam: the hidden parasite reservoir.
 Pham Vinh Thanh, Nguyen Van Hong, Nguyen Van Van, Carine Van Malderen, Valérie Obsomer, Anna Rosanas-Urgell, Koen Peeters Grietens, Nguyen Xuan Xa, Germana Bancone, Nongnud Chowwiwat, Tran Thanh Duong, Umberto D'Alessandro, Niko Speybroeck and Annette Erhart Malaria Journal (2015) 14:86 DOI 10.1186/s12936-015-0601-y
- Confirmed *Plasmodium vivax* Resistance to Chloroquine in Central Vietnam
 Pham Vinh Thanh, Nguyen Van Hong, Nguyen Van Van, Melva Louisa, Kevin Baird, Nguyen Xuan Xa, Koen Peeters Grietens, Le Xuan Hung, Tran Thanh Duong, Anna Rosanas-Urgell, Niko Speybroeck, Umberto D'Alessandro, Annette Erhart Antimicrob Agents Chemother 59:7411–7419. doi:10.1128/AAC.00791-15
- 4. Population Genetics of Plasmodium vivax in Four Rural Communities in Central Vietnam

Nguyen Van Hong, Christopher Delgado-Ratto, Pham Vinh Thanh, Peter Van den Eede, Pieter Guetens, Nguyen Thi Huong Binh, Bui Quang Phuc, Tran Thanh Duong,1 Jean Pierre Van Geertruyden, Umberto D'Alessandro, Annette Erhart, and Anna Rosanas-Urgell2. http://dx.doi.org/10.1371/journal.pntd.0004434

5. *P. vivax* morbidity after radical cure: a two-year cohort study in Central Vietnam

Pham Vinh Thanh, Nguyen Van Hong, Angel Rosas Aguirre, Nguyen Van Van, Mario A. Cleves, Nguyen Xuan Xa, Nguyen Thanh Thao, Tran Thanh Duong, Le Xuan Hung, Niel Hens, Anna Rosanas-Urgell, Umberto D'Alessandro, Niko Speybroeck, Annette Erhart

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