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Spread and Distribution of Drug Resistance and Compensatory Mutations in *Plasmodium falciparum*

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To my Father and Sisters

ABSTRACT

In 2010 there were an estimated 216 million cases of malaria worldwide. In Honduras there were ~9000 cases of which 88% were due to *Plasmodium vivax* mono-infection. Chloroquine (CQ) resistant *Plasmodium falciparum* have spread throughout the world curtailing its use. The only exception appears to be north of Panama where CQ reportedly remains efficacious and the drug of choice for treating both *P. falciparum* and *P. vivax*. Resistance to antimalarials is associated with specific genetic polymorphisms and recently a putative H⁺ pump (*pfvp2*) has been suggested to be linked to CQ resistant *P. falciparum*. The aim of this thesis was to identify resistance associated genetic polymorphisms in *P. falciparum* and *P. vivax* from Honduras and to describe the worldwide distribution of *pfvp2* polymorphisms and their correlation to CQ resistance.

Resistance associated genetic polymorphisms in *P. falciparum* and *P. vivax* multidrug resistance gene (*pfmdr1* and *pvmdr1*), dihydrofolate reductase (*pfdhfr* and *pvdhfr*), *P. falciparum* chloroquine resistance transporter (*pfcr1*), dihydropteroate synthase (*pfdhps*) and V-type H⁺ pyrophosphatase (*pfvp2*) were identified in field samples using PCR based methods. From Honduras, 37 *P. falciparum* and 64 *P. vivax* samples, collected from symptomatic patients were used. In addition, 50 samples from each of Colombia, Liberia, Guinea-Bissau, Tanzania, Iran, Thailand and Vanuatu were used. The samples represented a time period from 1978 to 2009 and areas with different prevalence of CQ resistant *P. falciparum*.

In samples from Honduras no genetic polymorphisms associated with CQ or sulphadoxine-pyrimethamine (SP) resistance were found in *P. falciparum*. In *P. vivax*, the CQ resistance associated *pvmdr1* 976F allele was found in 7/37 samples and the SP resistance associated *pvdhfr* 57L+58R alleles were found in 2/57 samples. When analysing the worldwide collection of samples, the *pfvp2* 405V, 582K and 711P haplotype was associated with the for CQ resistance essential allele, *pfcr1* 76T (P=0.007). Samples with *pfvp2* 405I and/or 582R and/or 711S were significantly more common in Liberia in 1978-1980 (P=0.01), all African countries (P=0.004) and all African countries + Honduras (P=0.01) compared to the rest of the world.

Our results suggest that *P. falciparum* and *P. vivax* in Honduras are sensitive to CQ and SP. However, small numbers of *P. vivax* had genetic polymorphisms suggesting a degree of tolerance to CQ and SP. The association between *pfcr1* 76T and the *pfvp2* 405V, 582K and 711P haplotype suggest that this haplotype is associated with CQ resistance. This is in line with previous research that has described increased expression of *pfvp2* during CQ exposure. The higher frequency of *pfvp2* 405I and/or 582R and/or 711S in CQ sensitive settings in Africa and Honduras suggests a larger variation in the *pfvp2* genome prior to the spread of CQ resistance further supporting the association between *pfvp2* and CQ resistance.

RESUMEN

Se estima que en el 2010 hubo 216 millones de casos de malaria en todo el mundo. En Honduras se reportaron alrededor de 9000 casos de los cuales 88% se debieron a mono-infecciones por *Plasmodium vivax*. El antimálarico cloroquina (CQ) se ha utilizado ampliamente, pero *Plasmodium falciparum* resistente se ha extendido por todo el mundo restringiendo su uso. La única excepción parece ser al norte de Panamá, donde se presume que CQ sigue siendo eficaz y es el medicamento de elección para el tratamiento de *P. falciparum* y *P. vivax*. La resistencia a los antimaláricos está asociada a polimorfismos genéticos específicos y recientemente se ha sugerido que una bomba putativa de H⁺ (*pfvp2*) está relacionada con resistencia a CQ en *P. falciparum*. El objetivo de esta tesis fue identificar polimorfismos genéticos asociados a resistencia en *P. falciparum* y *P. vivax* en Honduras y describir la distribución mundial de polimorfismos en *pfvp2* y su correlación con resistencia a CQ.

Los cambios genéticos asociados a resistencia en el gen de resistencia múltiple 1 (*pfmdr1* y *pvmdr1*) y dihidrofolato reductasa, (*pfdhfr* and *pvdhfr*) de *P. falciparum* y *P. vivax*, dihidropteroato sintasa (*pfdhps*); el transportador de cloroquina resistente (*pfcr1*) y V-type H⁺ pyrophosphatase (*pfvp2*) de *P. falciparum* fueron identificados en muestras de campo utilizando métodos basados en PCR. De Honduras, se usaron muestras de 37 *P. falciparum* y 64 de *P. vivax*, que fueron recolectadas de pacientes sintomáticos. Adicionalmente, 50 muestras de cada uno de los siguientes países: Colombia, Liberia, Guinea-Bissau, Tanzania, Irán, Tailandia y Vanuatu. Las muestras representaron el período tiempo de 1978-2009 y áreas con diferente prevalencia de *P. falciparum* resistente a CQ.

En las muestras de Honduras con *P. falciparum* no se encontraron polimorfismos asociados con resistencia a CQ o sulfadoxina-pirimetamina (SP). El alelo *pvmdr1* 976F asociado a resistencia a CQ en *P. vivax* se encontró en 7/37 y los alelos en *pvdhfr* 57L+58R asociados a resistencia a SP fueron encontrados en 2/57 muestras. Al analizar la colección mundial de muestras, el haplotipo de *pfvp2*, 405V, 582K and 711P fue asociado con el alelo esencial de resistencia a CQ, *pfcr1* 76T (P=0.007). Muestras con *pfvp2* 405I y/o 582R y/o 711S fueron significativamente más comunes en Liberia en 1978-1980 (P=0.001), todos los países Africanos (P=0.004) y todos los países Africanos + Honduras (P=0.01) comparadas con el resto del mundo.

Nuestros resultados sugieren que *P. falciparum* y *P. vivax* en Honduras son sensibles a CQ y SP. Sin embargo un pequeño número de *P. vivax* tenía polimorfismos genéticos lo que sugiere un grado de tolerancia. La asociación entre *pfcr1* 76T y el haplotipo de *pfvp2*, 405V, 582K y 711P sugiere que el haplotipo está asociado con resistencia a CQ. Esto apoya investigaciones previas que vincula la expresión del gen cuando se expone a CQ. La alta frecuencia *pfvp2* 405I, 582R y/o 711S en ambientes sensibles a CQ en África y América sugiere mayor variación en el genoma de *pfvp2* antes de la propagación de resistencia a CQ lo cual apoya más la asociación entre *pfvp2* y resistencia a CQ

LIST OF PUBLICATIONS

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- II. **Irina Tatiana Jovel**, Pedro Eduardo Ferreira, Rita Piedade, Maria Isabel Veiga, Maja Malmberg, Andreas Martenson, Akira Kaneko, Sedigheh Zakeri, Claribel Murillo Anders Bjorkman, Johan Ursing. Worldwide distribution of single nucleotide polymorphisms in *pfvp2* and their association with polymorphisms in *pfprt* and *pfmdr1*. Manuscript

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LIST OF ABBREVIATIONS

ACT	Artemisinin based combinations
CQ	Chloroquine
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DV	Digestive vacuole
G6PD	Glucose-6-dehydrogenase deficiency
IRS	Indoor residual spraying
LLINs	Long-lasting insecticidal nets
PCR	Polymerase chain reaction
<i>Pfcr1</i>	<i>P. falciparum</i> CQ resistance transporter
<i>Pfdhfr</i>	<i>P. falciparum</i> dihydrofolate reductase
<i>Pfdhps</i>	<i>P. falciparum</i> dihydropteroate synthase
<i>Pfmdr1</i>	<i>P. falciparum</i> multidrug resistance 1
<i>Pfmrp1</i>	<i>P. falciparum</i> multidrug resistance-associated protein
<i>Pfnhe1</i>	<i>P. falciparum</i> Na ⁺ H ⁺ exchanger 1
<i>Pfvp2</i>	<i>P. falciparum</i> V-type H ⁺ pyrophosphatase
<i>Pvcrt-o</i>	<i>P. vivax</i> chloroquine resistance transporter
<i>Pvdhfr</i>	<i>P. vivax</i> dihydrofolate reductase
<i>Pvdhps</i>	<i>P. vivax</i> dihydropteroate synthase
<i>Pvmdr1</i>	<i>P. vivax</i> multidrug resistance 1
RDT	Rapid diagnostic tests
RFLP	Restriction Fragment Length Polymorphism
SOPM	Standard operating procedures manual for malaria diagnosis by microscopy
SNP	Single nucleotide polymorphism
SP	Sulfadoxine-pyrimethamine
WHO	World Health Organization

Los Pobres

Por Roberto Sosa

Los pobres son muchos
y por eso
es imposible olvidarlos.

Seguramente
ven
en los amaneceres
múltiples edificios
donde ellos
quisieran habitar con sus hijos.

Pueden
llevar en hombros
el féretro de una estrella.

Pueden
destruir el aire como aves furiosas,
nublar el sol.

Pero desconociendo sus tesoros
entran y salen por espejos de sangre;
caminan y mueren despacio.

Por eso
es imposible olvidarlos.

The Poor

By Roberto Sosa
(Translated from the Spanish by Spencer Reece)

The poor are many
and so—
impossible to forget.

No doubt,
as day breaks,
they see the buildings
where they wish
they could live with their children.

They
can steady the coffin
of a constellation on their shoulders.

They can wreck
the air like furious birds,
blocking out the sun.

But not knowing these gifts,
they enter and exit through mirrors of blood,
walking and dying slowly.

And so,
one cannot forget them

1 BACKGROUND

1.1 GLOBAL MALARIA BURDEN

Malaria is an infectious disease caused by a parasite and transmitted to humans by female *Anopheles* mosquitoes. Malaria generally occurs in tropical and subtropical areas, is commonly associated with poverty and represents a major burden to economic and social development, costing an estimated sum of greater than US\$ 6 billion for the year 2010 [1, 2].

In 2010 it was estimated that 3.3 billion people were at risk of the disease. There were an estimated 216 million cases of malaria worldwide of which 91% were due to *P. falciparum*. Though *P. falciparum* is the most common malaria species, *P. vivax* is the most widespread. *P. ovale*, *P. malariae* and *P. knowlesi* are less common [3]. The African Region contributes the majority of malaria cases (81%) [2]. Worldwide, an estimated 1 238 000 deaths were attributed to malaria during 2010. Approximately 86% of malaria associated mortality occurred in children under 5 years of age and 91% of the deaths were in Africa [4].

In 2009 it was estimated that 2.85 billion people were at risk of *P. vivax* malaria infection. The majority of the cases are concentrated in Southeast Asia, Middle East and the Pacific [5]. In the Americas, *P. vivax* accounts for more than 70% of malaria cases [2]. *P. vivax* was previously considered as benign and self-limiting disease and therefore neglected from research. However, recent evidence has shown that infection with *P. vivax* also results in severe illness and death [6].

1.2. THE *PLASMODIUM* PARASITE

Plasmodium is a protozoan with more than 140 species that can infect birds, reptiles and mammals. Only five species have been shown to infect humans: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* [7]. The parasite was discovered in human blood by Alphonse Laveran in 1880. Later, in 1898, the parasite was observed in the mosquito by Ronald Ross that was able to complete a description of the life cycle.

1.2.1. Malaria Vectors

Malarial parasites are transmitted by the female *Anopheles* mosquito of which there are over 500 hundred species. Worldwide ~40 anopheline species have been documented to transmit parasites to humans with varying efficiency [7]. The female anopheles needs a blood meal and water site for laying eggs. Some vectors prefer blood meals from humans (anthropophilic) or from animals (zoophilic). Some of the vectors have the tendency to enter and rest inside houses (endophilic) and other rest outside (exophilic) after taking a blood meal. Most vectors have nocturnal feeding timings but habits vary with species [8]. *Anopheles* species with the greatest capacity to transmit *Plasmodium* are found in the Amazon (*An. darlingi*) and Africa (the species complex of *An. gambiae*). In Southeast Asia a highly efficient vector is *An. dirus* [7].

1.2.2. Life cycle

The female *Anopheles* mosquito injects sporozoites present in the saliva of the insect. Sporozoites infect the liver cells where they may remain dormant (hypnozoites) or produce schizonts and merozoites. When liver cells rupture, ~2 000 – 30 000 merozoites are released into blood and infect the erythrocytes. *P. ovale* and *P. vivax* infect immature erythrocytes whereas *P. malariae* infects mature erythrocytes. *P. falciparum* infects both. In the erythrocytes, the parasites mature into trophozoites. These trophozoites develop via schizonts into merozoites in erythrocytes which ultimately burst releasing the merozoites. Some of the merozoites transform into male and female gametocytes while others enter erythrocytes to continue the erythrocytic cycle. The gametocytes are ingested by the female mosquito. The female gametocyte transforms into ookinete that is then fertilized forming an oocyst in the gut. The oocyte produces sporozoites which migrate to the salivary gland and are ready to infect another host. The liver cycle takes 5-15 days (up to 3 years if hypnozoites from *P. vivax* or *P. ovale* exist) and the erythrocytic cycle takes 48 hours or 72 hours (*P. malariae*). Malaria can also be transmitted by transfusion and transplacentally.

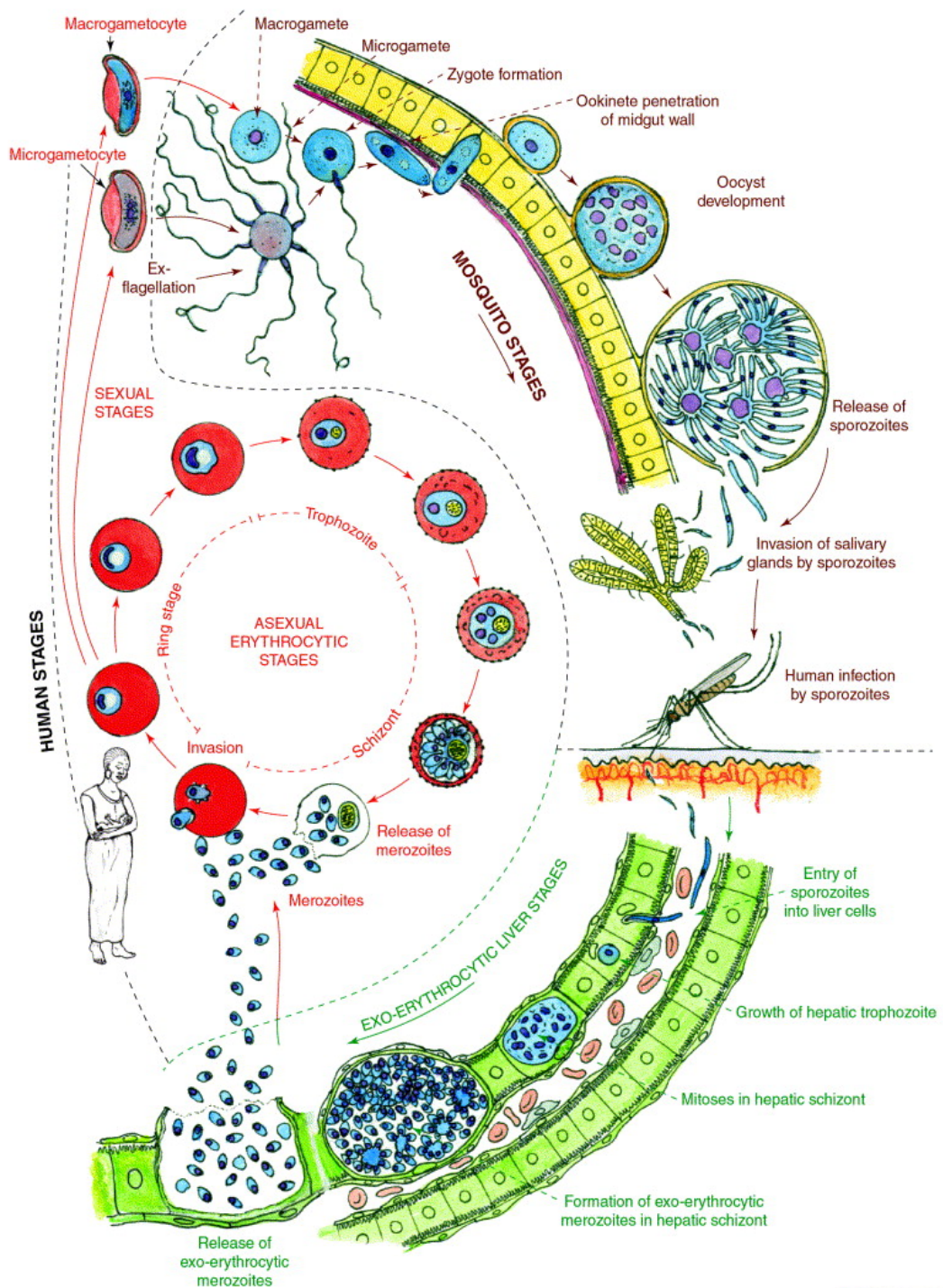


Figure 1 - The life-cycle of *Plasmodium falciparum*. The main phases in the liver and in the red blood cells (asexual and sexual erythrocytic stages) of the human host, and in the gut and in the salivary glands of the mosquito host are depicted. Reprinted from Trends in Parasitology [9], with permission from Elsevier.

1.3. CLINICAL PRESENTATION OF MALARIA

In clinical practice, malaria is defined as uncomplicated or severe. The first symptoms of malaria are nonspecific: a lack of a sense of well-being, headache, fatigue, abdominal discomfort, and muscle aches are followed by fever. The classic malarial paroxysms, in which fever spikes, chills, and rigors occur at regular intervals, are unusual and suggest infection with *P. vivax* or *P. ovale*. Most patients with uncomplicated infections have few abnormal physical findings other than fever, anemia, and in some cases palpable splenomegaly [7]. Anemia is common among young children living in areas with stable transmission, particularly where there is resistance to available antimalarials [10].

Severe malaria is an acute life threatening form of malaria with high (~ 10%) mortality in young children [11]. Severe malaria is commonly considered as a feature of *P. falciparum* though *P. vivax* can also result in severe disease and death. The characteristics of severe *P. vivax* are similar to those of severe *P. falciparum* malaria [6, 12, 13]. Studies from Indonesia, Papua New Guinea, Thailand and India where both species coexist, showed that 20-40% of malaria admissions were due to *P. vivax* mono-infections. In addition, in these mortality due to *P. vivax* (mono-infections) was 0.8-1.6%, similar to that observed with *P. falciparum* mono-infections (1.6-2.2%) [14]. The underlying mechanisms of severe manifestations in *P. vivax* are not fully understood.

1.4. MALARIA CONTROL

Progress in shrinking the malaria map has been remarkable. There are 79 countries that have eliminated malaria since 1954 and the proportion of the world's population who live in malaria-endemic regions has decreased from 70% to 50%. [15]. Malaria eradication was first undertaken by the WHO between 1955 and 1969. The malaria eradication programme used vector control and effective treatment as primary tools to manage malaria. Despite many gains made during the program, the eradication effort was terminated in 1969. Among the reasons for the stop of the program were widespread resistance to available insecticides, wars, massive population movements, difficult to obtain funding and finally the emergence of CQ resistance in the 1960s.

In the last decade there has been renewed interest and action to support malaria research, control, and eradication. [7, 16]. The Current malaria control strategy was launched in the early 2000s. The principle aims are to prevent malaria and improve

case management as described below. The strategy appears to have been successful and [2, 17] coincident with the introduction of control measures including artemisinin based combinations (ACTs), the malaria attributed mortality decreased from approximately 1 817 000 in 2004 to 1 238 000 in 2010 [4].

2.

1.4.1. Vector control

The goals of malaria vector control include protection of individual people against infective malaria mosquito bites and reduction of the intensity of local malaria transmission at community level. This should be achieved by reducing the longevity and density of the vector and human-vector contact. The two most powerful and most broadly applied interventions are long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS) [2]. LLINs have been shown to decrease morbidity and mortality in various malaria transmission settings thereby having a major impact on the malaria burden [18]. IRS involves application of insecticides to the inner surfaces of dwellings, where many vector species of *Anopheles* mosquito tend to rest after taking a blood meal. This strategy has also proved to effectively reduce malaria transmission in areas with low and variable/seasonal transmission [19]. Worryingly, a recent study in Senegal showed that the prevalence of insecticide tolerant mosquitoes and *P. falciparum* incidence increased 27-30 months after the introduction of LLINs [20].

1.4.2. Diagnosis of malaria

Prompt parasitological confirmation by microscopy or rapid diagnostic tests (RDTs) is recommended before treatment is started [2]. In settings with limited health facility access, diagnosis and treatment should be provided at community level through a programme of community case management [12].

1.4.3. Treatment of uncomplicated malaria

P. falciparum has developed resistance to CQ, followed by SP followed by mefloquine when used as monotherapy. The WHO therefore recommends artemisinin based combination therapy (ACT) to treat uncomplicated *P. falciparum* malaria [21, 22]. In pre-elimination or elimination programs a single dose of primaquine is also recommended [2]

The choice of the ACT should be based on the efficacy of the combination in the country or area of intended use. Artemisinin and its derivatives should not be used as oral monotherapies for the treatment of uncomplicated malaria as poor adherence to the required 7 days of treatment results in partial clearance of malaria parasites which will promote resistance to this critically important class of antimalarials [12].

P. vivax malaria should be treated with CQ in areas where CQ is effective. When CQ resistance is present, an appropriate ACT should be used. In order to prevent relapses a 14 day course of primaquine should be used in combination with both CQ and ACT.

In Honduras, CQ is recommended for treatment of uncomplicated *P. falciparum* and *P. vivax* infection. In addition, primaquine is used for treatment of *P. falciparum* gametocytes and *P. vivax* hypnozoites [23, 24]. These drugs appear to remain effective despite being used for six decades and despite the spread of CQ resistance across most of the rest of the world. In addition CQ is cheap, well tolerated and available.

1.4.4. Treatment of severe malaria

Severe malaria should be treated with a parenteral artemisinin derivatives (artesunate and artemether) followed by a complete course of an effective ACT as soon as the patient can take oral medications [12]. If artemisinin derivatives are not available parenteral quinine or quinidine can be used as alternative. Where complete parenteral treatment of severe malaria is not possible patients should be given pre-referral treatment and immediately be referred to an appropriate facility for further treatment. Options available for pre-referral treatment are: Rectal or intramuscular artesunate, intramuscular quinine or arthemeter [12].

1.5. ANTIMALARIAL DRUGS DISCUSSED IN THIS THESIS

1.5.1. Quinolines and related compounds

The first effective chemotherapy to treat malaria was extracts from the Cinchona bark tree. The extract was imported from South America to Europe in the mid 17th century where it became popular for the treatment of fevers including malaria. In 1820 pure QN was isolated from the Cinchona bark and replaced the extract in the treatment of malaria [21]. The basic quinoline ring structure has provided a group of synthetic antimalarials (chloroquine, amodiaquine, piperaquine, mefloquine and primaquine) collectively named quinolines. Based on more loosely related ring systems the antimalarials halofantrine and lumefantrine have also been synthesized [25].

CQ has been used extensively for the treatment and prevention of malaria since 1947. It is safe, cheap and is estimated to have saved countless millions of lives. As such it is one of the most successful drugs ever produced [26]. Widespread resistance has now rendered it virtually useless against *P. falciparum* infections in most parts of the world but not north of the Panama Canal. It is still efficacious for the treatment of *P. vivax*, *P. ovale* and *P. malariae* infections in most parts of the world.

Amodiaquine (AQ) is a potent blood schizonticide that was developed in the late 1940s and has been used for the treatment of uncomplicated malaria particularly in Africa [27]. When used for malaria prophylaxis AQ cause neutropenia and for mainly that reason it was not used for many years. However, it has been revived as part of an ACT.

Mefloquine was introduced to treat patients with CQ resistant parasites. It was used as monotherapy in areas of low transmission malaria [28]. The drug is effective against all forms of malaria, [29] however due to the spread of resistance it is now principally used in combination with artesunate to treat *P. falciparum* in Southeast Asia (Cambodia, Malasia, Myanmar, Thailand and Vietnam) and South America (Bolivia, Brazil, Colombia, Peru and Venezuela) [2]

Lumefantrine is a blood schizonticide that was first synthesized in China and first mentioned in scientific literature outside China in 1990. It is only available as an oral preparation coformulated with arthemeter. This ACT is highly effective against *P. falciparum* and a corner stone of ACT in Africa.

The quinolines do not eradicate hypnozoites unlike primaquine that was developed during the Second World War. Primaquine is effective against intrahepatic forms of all types of malaria parasite. It is gametocytocidal against *P. falciparum* and has significant blood stages activity against *P. vivax* (and some against asexual stages of *P. falciparum*). The mechanism of action is unknown. The hemolytic effect in patients with glucose-6-dehydrogenase deficiency (G6PD) and gastrointestinal intolerance limits its use.

1.5.1.1.1. Quinoline mechanism of action

CQ is a weak base that moves rapidly across cell membranes and accumulates in the acidic environment of the digestive vacuole because it becomes protonated [30]. In the digestive vacuole CQ disrupts the detoxification of heme when haemoglobin is digested by the parasite [31]. This results in heme complexes that are lethal to the parasite [32]. The mechanism of CQ activity against blood stages of *P. vivax* remains unknown [33]. Quinine, amodiaquine and piperazine are believed to have similar modes of action to that of CQ [34]. Mefloquine, halofantrine and lumefantrine have also been shown to inhibit the detoxification of heme but they also appear to target other process in the parasite [35, 36].

1.5.2. Antifolates

The antifolates are compound that bind to enzymes necessary for parasite folate biosynthesis. The principal antifolate drugs used against malaria are the combination sulphadoxine-pyrimethamine (SP).

Sulfadoxine is a structural analogue and competitive antagonists of p-aminobenzoic acid. It potentiates the schizontocidal effect and improves clinical response of pyrimethamine when treating *P. falciparum* infection [37-39]. In 1951 pyrimethamine was shown to be effective for the treatment of *P. falciparum* [40, 41]. It inhibits dihydrofolate reductase (*pfdhfr*) thus indirectly blocking the synthesis of nucleic acids in the malaria parasite. It is a slow-acting blood schizontocide and is possibly active against pre-erythrocytic forms. Furthermore, it inhibits sporozoite development in the mosquito vector. During the 1950–1960s, pyrimethamine was mainly used for prophylaxis against *P. falciparum* infection or for mass drug administration because CQ was effective in all endemic regions [42-44]. In the late 1960s, an antifolate

combination of sulfadoxine and pyrimethamine, SP, was first introduced in Thailand where the frequency of CQ-resistant *P. falciparum* infections had reached an unacceptable level.

1.5.3. Artemisinin

Artemisinin, also known as qinghaosu, is a semisynthetic drug extracted from the leaves of *Artemisia annua* (sweet wormwood). It has been used in China for the treatment of fever for over a thousand years and artemisinin derivatives are now the cornerstone of antimalarial combination therapy. It is a potent and rapidly acting blood schizontocide and is active against all *Plasmodium species*. It has an unusually broad activity against asexual parasites. Furthermore it kills *P. falciparum* gametocytes [45]. The mechanism of action of the artemisinin is not fully understood [46]. Commonly used artemisinin derivatives are dihydroartemisinin, artemether, and artesunate. The two latter derivatives are *in vivo* converted back to dihydroartemisinin. The 5 ACTs currently recommended are arthemeter + lumefantrine, artesunate + amodiaquine, artesunate + mefloquine, artesunate + sulphadoxine-pyrimethamine and dihydroartemisinin + piperaquine [12]

1.6. ANTIMALARIAL DRUG RESISTANCE

One definition of clinical drug resistance is “the ability of a parasite strain to survive and/or to multiply despite the administration and absorption of antimalarial drug in the dose normally recommended.” Antimalarial drug resistance is not necessarily the same as malaria “treatment failure”, which is a failure to clear malarial parasitaemia and/or resolve clinical symptoms despite the administration of an antimalarial. So while drug resistance may lead to treatment failure, not all treatment failures are caused by drug resistance [47].

Drug resistance in malaria does probably not arise in a single step, but as a long process during which the parasites become gradually more and more tolerant to the drug in question. This is believed to be achieved through the gradual accumulation of genetic changes as discussed below. The changes alter the natural function of a specific protein that in turn may require additional compensatory genetic changes. Eventually a fully resistant and fit parasite emerges. For *P. falciparum* this process typically takes 1-12 years.

The greatest problem with drug resistance occurs with *P. falciparum* but CQ resistant *P. vivax* is a developing problem. There are very few reports although there have also very few studies on drug resistant *P. malariae* and *P. ovale*. Of greatest concern at the moment are recent reports of *P. falciparum* that are resistant/tolerant to artemisinin and to ACT [47-52].

1.6.1. Development and spread of chloroquine resistance

P. falciparum CQ resistance took a long time to develop (>10 years) [22] and only appears to have arisen 5 times [53]. In the late 1950s, CQ resistant *P. falciparum* was identified at the Thai-Cambodian border and simultaneously in two different locations in South America (Colombia and Venezuela) [21]. In Pacific regions CQ resistance was first reported 1959–1961 [54]. Resistance then spread to eastern parts of the Pacific region between 1976 and 1980. By the mid 1970s CQ resistance was spread in all Southeast Asia. All endemic areas in South America were affected by 1980 and almost all of Asia and the Pacific by 1989 [22]. In Africa CQ resistance first appeared on the east coast in 1978 [55, 56]. During the early 1980s it spread throughout East Africa and by the early 1990s, CQ resistant *P. falciparum* became a serious emerging problem in many West African countries [53]. CQ resistance has been reported from wherever *falciparum* malaria is endemic, except Central America [47, 57].

P. vivax is still generally sensitive to CQ although sensitivity is decreasing in some areas. CQ resistant *P. vivax* was first described in 1989 when it was reported from Papua New Guinea [58]. Subsequently other reports from Indonesia confirmed those findings [59-61]. By 2002 there were reports from Malaysia, Myanmar, Vietnam, India and Iran [62-66] and by 2009 from Turkey and South Korea [67, 68]. In South America CQ resistance was first reported in 1996 from French Guyana [69]. By 2003 Brazil, Colombia and Peru had also reported cases of CQ resistant *P. vivax* [70-72]. First reports from Africa are from Ethiopia and Madagascar in 2008 [73, 74].

1.6.2. Development and spread of sulphadoxine-pyrimethamine resistance

SP resistant *P. falciparum* were described at the Thai-Cambodia border in 1967, the same year that SP was introduced. SP resistance subsequently spread to other regions in Southeast Asia [22]. In 1996, high-level resistance was found simultaneously in a large part of Southeast Asia, Southern China and the Amazon Basin. Lower degrees and

frequencies of resistance were observed on the Pacific coast of South America [22]. Sensitivity to SP in Africa started to decline in the late 1980s. It is not known when pyrimethamine resistance was imported to Africa although a study indicates that the Asian origin mutant had arrived in Kenya by 1987. The picture is further complicated by evidence of an indigenous evolution in Ghana and Kenya [75].

In early reports from the 1950's *P. vivax* appeared to be resistant to SP. Recently it was suggested that the early perception of poor efficacy may have been a product of confusion with the failure of SP to prevent relapse [76]. However, clinical failure following SP treatment has now been reported from Papua New Guinea and Indonesia in 1992 [77, 78]. By 2005 resistance had also been reported from, Myanmar, Vietnam, Vanuatu and India [79, 80].

1.6.3. Artemisinin resistance

In 2005, there was a report of reduced *in vitro* susceptibility to arthemeter in isolates from French Guyana and Senegal [81]. *In vivo* artemisinin resistance or perhaps more correctly tolerance is characterized by a slower rate of parasite clearance. This has been described from Western Cambodia, Western Thailand, Southeastern Burma and Southeastern Vietnam. A recent report from Western Thailand describes how the proportion of *P. falciparum* with reduced artesunate susceptibility increases over time. [50, 51, 82-84]

1.7. RESISTANCE ASSOCIATED GENES

Resistance appears to be caused by a change in the structure, function or quantity of a protein. The change in the protein is in turn mediated by genetic changes such as single nucleotide polymorphisms (SNP) or gene amplifications. An alteration in the structure of a protein may prevent the drug from binding to its target as in SP resistance [75, 85]. An alternative mechanism is to enhance or block a transport proteins function so that a drug is removed from its site of action (e.g. CQ efflux from the DV) or prevented from entering into its site of action (e.g. import of CQ or QN in to DV). Changes in two genes may act in combination to produce a specific phenotype [86].

1.7.1. Quinolines

The two principle genes that appear to be involved in quinoline resistance are *pfcr1* and, *pfmdr1*. Both are located in the membrane of *P. falciparum*'s digestive vacuole. Both are believed to be transporters and different SNPs hinder or enable the transport of different quinolines. The net effect is most probably to decrease the concentration of the various drugs at their various sites of action. Molecular markers of drug resistance in *P. vivax* have been based on the analysis of *P. falciparum* orthologs *pvcrt-o*, *pvmadr1* genes

1.7.1.1. *P. falciparum* chloroquine resistance transporter – *pfcr1*

More than 40 years were necessary to go from clinical recognition of CQ resistance to the molecular basis of the phenomena which was unravelled in 2000 when *pfcr1* was identified [87]. *Pfcr1* is located in the membrane of the DV and a *pfcr1* K76T SNP has been shown to be essential for CQ resistance *in vivo* and *in vitro* [88-91]. Since then it has been shown that the replacement of Lysine (K) with Threonine (T) removes a positive charge enabling *pfcr1* to transport protonated CQ down its electrochemical gradient, out of the DV [92, 93]. The net result is lower non toxic CQ concentrations in the DV and continued parasite growth. Specific haplotypes at positions 72-76 are linked to the regional evolution of resistance [53].

Pfcr1 has also been shown to influence susceptibility to other antimalarial drugs. Amodiaquine resistance is linked to *pfcr1* 76T just as CQ whereas allelic exchange experiments has linked *pfcr1* 76T to decreased sensitive to mefloquine, artemisinin and quinine [89, 94, 95]. Furthermore, K76 has been linked to reduced susceptibility to lumefantrine [96] and 76I showed increased sensitivity to quinine but reduced sensitivity to quinidine *in vitro* [94].

1.7.1.2. *P. falciparum* multidrug resistance 1 – *pfmdr1*

Before the discovery of *pfcr1* most attention was given to *pfmdr1* [97]. The first *pfmdr1* polymorphism that was correlated to drug resistance was gene copy number [98-100]. Multiple *pfmdr1* copies were shown to be a molecular marker of *in vitro* [101] and *in vivo* [49] mefloquine resistance. Decreased *in vitro* susceptibility to lumefantrine, halofantrine, quinine and artemisinin has also been linked to amplifications. Furthermore, amplifications have been associated with an increased risk of failure following arthemeter-lumefantrine treatment (4 doses) [52, 102].

Drug resistance associated SNPs in *pfmdr1* include N86Y, Y184F, S1034C, N1042D, F1226Y and D1246Y. Various constellations of these SNPs have been shown to modulate the level of drug resistance/tolerance to quinine, chloroquine, mefloquine, halofantrine and lumefantrine [48, 103, 104]. SNPs in *pfmdr1* do not confer CQ resistance [103, 105] however *pfmdr1* N86Y together with *pfcr1* K76T has been associated with high levels of CQ resistance [106, 107].

1.7.1.3. *P. falciparum* V-type H⁺ pyrophosphatase – *pfvp2*

Pfvp2 is a novel class of H⁺ pump found in plants and some protozoa [108-112]. *Pfvp2* is located in the DV membrane and increased transcription of *pfvp2* has been observed *in vitro* when *P. falciparum* are exposed to CQ [112] and lumefantrine [96]. Specifically a ten-fold up-regulation of *pfvp2* was observed when the CQ resistant (*pfcr1* 76I) *P. falciparum* 106/1 clone was exposed to CQ but no up-regulation was seen with the CQ sensitive 106/1 (*pfcr1* 76K) clone. A two-fold up-regulation of *pfvp2* was seen when lumefantrine tolerant *P. falciparum* V1S (*pfcr1* 76K) clone was exposed to lumefantrine. As described above CQ resistance involves the removal of protonated CQ from the DV. This is likely to cause a loss of H⁺ that needs to be replaced if the DV is to remain acidic. The up-regulation of *pfvp2* suggests that it could be involved in maintaining the H⁺ balance in the parasite DV and to compensate for H⁺ loss caused by removal of protonated CQ [112].

1.7.1.4. *P. vivax* chloroquine resistance transporter – *pvcrt-o* and *P. vivax* multidrug resistance 1 - *pvmdr1*

In *P. vivax* the mechanism of CQ activity against blood stages remains unknown. The search for molecular markers of resistance in *P. vivax* has focused on the orthologs *P. vivax* multidrug resistance gene 1 (*pvmdr1*) and chloroquine resistance transporter gene (*pvcrt-o*) [33]. However, no association between mutations in the *pvcrt-o* and CQ *in vivo* or *in vitro* response has been found [113, 114]. The *pvmdr1* 976F allele has been associated with reduced susceptibility to CQ and increased susceptibility to mefloquine and artesunate in Southeast Asia [115]. Amplifications of *pvmdr1* have been associated with reduced susceptibility to mefloquine and artesunate [115-117].

1.7.2. Dihydrofolate reductase–*dhfr* and dihydropteroate synthase–*dhps*

The *dhfr* coding region was cloned from *P. falciparum* in 1987 [118] and the *P. vivax* gene was identified in 1998 [119]. The *dhfr* genes from both species are ~66% identical and the active site regions are strongly conserved [120]. The gradual acquisition of resistance associated SNPs (N51I, C58R, S108N and I164L) in *pfdhfr* results in increasing levels of drug tolerance [121]. The triple dihydrofolate reductase (*pfdhfr*) haplotype N51I/C59R/S108N has been associated with SP treatment failure and when dihydropteroate synthase (*pfdhps*) SNPs G437A and K540E are added, highly SP resistant *P. falciparum* are generated [116, 122-125]. Twenty single nucleotide polymorphisms have been described in *P. vivax* dihydrofolate reductase (*pvdhfr*) including F57L, S58R, T61M and S117N/T that correspond to codons 50, 51, 59 and 108 in *pfdhfr*, respectively [79, 126-128]. *Pvdhfr* S58R and S117N result in decreased binding of pyrimethamine [120] and quadruple (F57L, S58R, S117N and I173L) SNPs have been associated with SP treatment failure [79, 126, 127]. In the *pvdhfr* gene small in-frame insertions and deletions (indels) are frequently observed.

2. AIMS OF THE THESIS

Overall Aim:

The overall aim is to identify genetic changes associated with antimalarial resistance

Specific aims:

Paper I: Determine the proportion of chloroquine and SP resistance associated genetic polymorphisms in *P. falciparum* and *P. vivax* collected in Honduras.

Paper II: Identify genetic changes in *pfvp2* and to describe their worldwide prevalence and association with polymorphisms in *pfcr1* and *pfmdr1*.

3. MATERIALS AND METHODS

3.1. STUDY SITES

For study I blood samples were collected from the Hospital Escuela that is a teaching hospital in Distrito Central-Tegucigalpa, the regional hospitals in Trujillo, La Ceiba and Juticalpa, two primary health centres in Puerto Lempira and Iriona and one regional laboratory in Juticalpa. For study II Blood samples from 385 patients collected in the following countries were analysed; Liberia (1978-1981), Vanuatu (2002), Guinea Bissau (2001-2004), Honduras (2004-2009), Tanzania (2008), Iran (2001-2002), Colombia (1999-2001) and Thailand (2002-2008).

3.1.1. Honduras

3.1.1.1. Malaria distribution

In Honduras the transmission is seasonal and *A. albimanus* and *A. darling* are the two main vectors responsible for transmitting malaria. They transmit the parasite during the rainy and dry seasons, respectively. Before the 1980s most of the malaria cases were in the south of the country due to the high density of anopheles and the extensive rice crops. With the collapse of these crops in the Pacific and the rise of agribusiness (African palm, banana, citrus and maquilas) on the Atlantic coast in the 1990s the malaria cases moved to the North of the country [129].

In 2010 there were 9,078 reported malaria cases. *P. vivax* mono-infection accounted for 88% and 12% were due to *P. falciparum* mono-infection and mixed *P. vivax* and *P. falciparum* infections. Typically 95% of the cases are reported from the five Atlantic regions (of 18 national health regions) of which Gracias a Dios region reports 87% of *P. falciparum* in the country [24]

3.1.1.2. Malaria Diagnosis

Malaria cases are mainly reported by microscopy and through passive case detection. Since 2006 the country has a Standard operating procedures manual for malaria diagnosis by microscopy (SOPM) [130]. This SOPM has proved to be a valuable tool at all levels of diagnostics when microscopy is available. Thick and thin smears are made and stained using Giemsa. Slides are viewed under X100 magnification. Trained

laboratory technician examine at least 100 microscopy fields before considering a sample negative. Microscopy is quality controlled at the National Malaria Laboratory where microscopists routinely re-examine all positive and approximately 10% of negative slides.

3.1.1.3. Sample collection

Samples were collected from patients that sought medical attention at Hospital Escuela between 2004 and 2006 and during 2009 at all other sites. At the Hospital Escuela sample collection was considered to be part of routine malaria surveillance and did not involve additional sampling or collection of patient data. At the other health facilities patients who sought medical attention and were diagnosed with malaria were invited to participate in the study after written informed consent.

3.1.2. Colombia

Malaria transmission presents an endemic/epidemic condition that maintains unstable endemic transmission levels throughout the country. Malaria is caused mainly by *P. vivax* and *P. falciparum* and occasionally by *P. malariae* [131]. Samples were collected between 2001 and 2005 and were provided by Centro Internacional de Entrenamiento e Investigaciones Medicas (CIDEIM) in Cali, Colombia. When the samples were collected the national drug policy was amodiaquine + SP [131].

3.1.3. Liberia

Malaria in Liberia is considered to be holoendemic. Samples were collected between 1978 and 1981 and in 1985 it was reported that malaria was caused mainly by *P. falciparum* but *P. malariae* and *P. ovale* were present [132, 133]. At the time of sample collection the official antimalarial drug policy was CQ.

3.1.4. Guinea-Bissau

Malaria is generally considered to be meso or hyperendemic in Guinea-Bissau. The main malaria species is *P. falciparum*. From December 2001 to May 2004, blood samples were collected as part of a clinical trial in the semi-urban area of Bandim on the outskirts of Bissau [134]. The national policy for the treatment of uncomplicated malaria at the time was CQ.

3.1.5. Tanzania

Malaria is transmitted throughout the year with seasonal peaks during rainfalls in March-May and October-December. The main malaria species is *P. falciparum*. The survey, used for study II, was conducted in Fukayosi primary health care centre in 2008. The Fukayosi village is located in Bagamoyo district on mainland, Tanzania. Artemether + lumefantrine was used as first line treatment in Tanzania during the sample collection.

3.1.6. Iran

Malaria transmission occurs during the whole year and *P. falciparum* and *P. vivax* are both causes of malaria in the country. Samples were collected at the Chababar City Public Health Department in the Sistan-Baluchistan province of Iran. The study was conducted from April 2001 to March 2002 and the first line treatment was CQ according to national guidelines [135].

3.1.7. Thailand

Malaria in Thailand is endemic especially in the forest regions and the border areas. Both *P. falciparum* and *P. vivax* are present in the country. Thailand's Western border with Burma/Myanmar and eastern border with Cambodia are epicentres of emerging antimalarial drug resistance. *P. falciparum* isolates were collected from 2002 to 2008 from patients in Mae Sot, in the Tak Province. The isolates were provided by the Shoklo Malaria Research Unit. Artesunate + mefloquine was used as the first line treatment for uncomplicated confirmed *P. falciparum* during sample collection [136].

3.1.8. Vanuatu

Vanuatu is a South Pacific archipelago made up of over 80 islands, each with varying levels of malaria. Malaria transmission is perennial but seasonal in intensity [137]. *P. falciparum* and *P. vivax* both occur in Vanuatu. Samples were collected in Ambae Island in 2002. At the time CQ + SP was the first line treatment for uncomplicated *P. falciparum* malaria.

3.2. ETHICAL CONSIDERATIONS

Studies conducted in Guinea-Bissau, Honduras, Iran and Thailand were approved by local authorities as published elsewhere [57, 134, 135, 138]. Studies conducted in Tanzania, Vanuatu and Colombia were approved by the National Institute for Medical Research Tanzania (NIMR/HQ/R.8A/Vol. IX/344), the ethical committee in Tokyo Women's Medical University (Approval 2004-7-05/No. 69) and the Ethical Committee of CIDEIM, Cali, Colombia, respectively. Molecular analyses were approved by the Stockholm regional ethics board (reference number 2011/832-32/2).

3.3. MOLECULAR ANALYSIS

3.3.1. Sample storage, DNA extraction and amplification

DNA was extracted from the filter papers using an ABI Prism[®] 6100 Nucleic Acid Prep Station (Applied Biosystems, Fresno, CA) according to the manufacturer's instructions with minor modifications [139]. Extracted DNA was stored at -20°C.

3.3.2. Restriction Fragment Length Polymorphism (RFLP)

Previously described multiplex PCR-RFLP methods were used to identify the following SNPs; *pfert* K76T, *pfmdr1* N86Y, *pfdhfr* N51I, C59R, N108T/S and *pfdhps* G437A and K540E [140, 141]. *Pfvp2* SNPs V405I and P711S were identified using nested PCR amplifications followed by restriction. A first set of primers were used to amplify nucleotide 1112 to 2260 to include all 3 described SNP's. Two primer pairs were then used to amplify fragments that included codons 405 and 711. Primers were designed using Primer Express software (Applied Biosystems, Fresno, CA, USA) based on published sequence of *P. falciparum* (Gene Bank Accession No. AF283528). All reactions included Taq polymerase reaction buffer, magnesium chloride, dNTPs, forward and reverse primer pair and GoTaq[®] DNA polymerase. Restriction enzymes (New England Biolabs) used to analyse SNP's 405 and 711 were AseI and DpnI. PCR products were resolved on 2% agarose gels (Amresco, Solon, OH). All gels were stained with ethidium bromide and visualized under UV transillumination (GelDoc[®], Biorad, Hercules, Ca, USA).

3.3.3. Sequencing

Pfert 72-76 haplotypes were identified as described elsewhere [142]. For identification of *pfvp2* K582R allele new primers (nucleotides 1484-1929) were designed for a nested PCR amplification followed by sequencing. Previously described nested PCRs were

used to amplify codons 917 to 1118 of *pvmdr1* and codons 1 to 238 of *pvdhfr* [115, 143]. *Pvmdr1* and *pvdhfr* SNPs were then identified by sequencing. PCR products were purified and sequenced commercially (Macrogen Inc. Seoul, Korea).

The Sequencher™ software version 4.6 (Gene Codes Corporation, Ann Arbor, MI) was used for sequence analysis. The *P. falciparum* 3D7 clone sequence obtained from NCBI database (*pfcr1* Gen-Bank Accession no. NC_004328 and *pfvp2* Gene Bank Accession No. AF283528) was used as reference for *pfcr1* and *pfvp2*. For *pvdhfr* the *P. vivax* ARI/Pakistan isolate sequence (Gen-Bank accession no. X98123) and for *pvmdr1* the *P. vivax* Sal-1 isolate sequence (Gen-Bank accession no. AY618622) were used as references.

3.3.4. Real-Time PCR

Pfmdr1 and *pvmdr1* copy numbers were determined using real time PCR (ABI Prism® 7000 Sequence Detection System) as previously described [49, 144]. All samples were run in triplicate. For *pfmdr1* 3D7, D10 and K1 clones were used as single copy calibrators and FCB and Dd2 were multiple copy controls. *Pvmdr1* single and double copy calibrators were created by the insertion of *pvmdr1* nucleotides 2751-3354 and *pvbtubulin* nucleotides 860-1056 in the pCR2.1 vector using the TOPO TA-cloning kit (Invitrogen, Carlsbad, CA) at 1:1 and 2:1 proportions, respectively. The sample copy numbers were calculated using a comparative threshold method ($\Delta\Delta C_t$). Copy number >1.6 and copy number >2.6 were defined as 2 and 3 copies of *pfmdr1*, respectively. Assays were repeated if the following results were obtained: copy number 1.3-1.6 and 2.3-2.6 or Ct value >35 or standard deviation value >0.5

3.4. STATISTICS

Data were entered, validated and analysed on Microsoft Excel 2003. Allele proportions were calculated by dividing the number of samples with a certain allele by the number of samples with an identifiable allele at that position. Thus mixed infections contributed to the proportion of both alleles. When the association between *pfvp2* alleles and alleles in *pfcr1* and *pfmdr1* were assessed patient samples with mixed *pfcr1* K76T and/or *pfmdr1* N86Y alleles were excluded. When the association between the number of patient samples with SNPs in *pfvp2* and *pfcr1* K76T and *pfmdr1* N86Y were assessed (Table 2) only patient samples in which all alleles had been successfully identified were used. Associations were determined using Fishers Exact test using StataCorp 12.

4. RESULTS AND DISCUSSION

4.1. POLYMORPHISMS IN *P. FALCIPARUM*

The *pfcr* 72-76 haplotype was sequenced in samples from 8 different countries and 3 (of 5) different CQ resistance associated haplotypes were found. The haplotypes found were in line with previous reports and represent CQ resistance originating from Colombia (SVMNT), Papua New Guinea (SVMNT) and Thailand (CVIET, which subsequently spread to Africa). The CQ sensitive haplotype found in Liberia prior to the arrival of CQ resistance was the same as that found in CQ sensitive parasites in Guinea-Bissau where CQ resistance has never gained the upper hand. In Honduras the same CQ sensitive haplotype was also found. This is to our knowledge the first report on these haplotypes from Mesoamerica and West Africa prior to the arrival of CQ resistant *P. falciparum*.

The proportion of *P. falciparum* with *pfcr* 76K was significantly higher in Liberia (50/50) and Honduras (30/30) compared to all other countries ($P < 0.001$). The proportion was also higher in Guinea-Bissau (36/50) and Tanzania compared to Colombia, Iran, Thailand and Vanuatu ($P < 0.001$). Irrespective of whether Liberia was included (119/150) or not (69/100), the proportion of *pfcr* 76K was significantly higher ($P < 0.001$) in African countries compared to Asia (2/99) or South America (0/50). Allele frequencies in each country are presented in table 1. Finding a higher proportion of *pfcr* 76K in Guinea-Bissau and Tanzania is in line with previous studies. A probable explanation is that *pfcr* 76T is associated with a loss of fitness in Africa. This causes the prevalence of the SNP to rapidly decrease when the CQ pressure decreases [145-147]. Considering this loss of fitness it is interesting to note that the *pfcr* 76T prevalence remains fixed in Thailand and Colombia where CQ resistance developed unlike Africa where CQ resistance was imported.

Table 1 – Frequencies of polymorphisms in *pfvp2*, *pfprt* 76 and *pfmdr1*

Country	<i>Pfvp2</i>			<i>pfprt</i>		<i>pfmdr1</i>		<i>Pfmdr1</i> CN ^d		
	405I	582R	711S	K76 ^a	76T	N86	86Y	1	2	3
Liberia	3/48	1/48	4/49	50/50	0/50	46/47	2/47	30/30		
Guinea Bissau	3/50	1/50	3/50	36/50	16/50 ^b	28/50	24/50	50/50		
Tanzania	0/49	1/50	2/48	33/50	17/50 ^b	34/50	16/50	46/46		
Iran	1/50	1/50	1/50	2/50	49/50 ^c	13/50	37/50	36/36		
Thailand	1/49	0/49	1/49	0/49	49/49 ^b	49/49	0/49	26/49	18/49	5/49
Vanuatu	0/31	0/32	0/38	4/38	38/38 ^c	6/32	31/32	9/9		
Honduras	2/30	0/30	1/30	30/30	0/30	30/30	0/30	28/28		
Colombia	0/46	0/46	0/50	0/50	50/50	50/50	0/50	38/44	8/44	

^a*Pfprt* 72-76 haplotype was CVMNK ^b*pfprt* 72-76 haplotype was CVIET. ^c*pfprt* 72-76 haplotype was SVMNT. ^dCN: Copy number.

The Frequencies and geographic distribution of *pfvp2* V405I, K582R and P711S are shown in Table 1. The *pfvp2* 405V, 582K and 711P haplotype was associated with *pfprt* 76T (P=0.007) and the *pfprt* 76T + *pfmdr1* 86N haplotype (P=0.025) as shown in Table 2. As *pfprt* 76T is essential for CQ resistance these results suggest that the *pfvp2* 405V, 582K and 711P haplotype is associated with CQ resistance. *Pfvp2* up-regulation has been shown to occur in *P. falciparum* with the CQ resistant allele *pfprt* 76I but not with the CQ sensitive allele 76K under CQ pressure [112]. This was proposed to be due to a need for increased H⁺ transport into the parasite DV to compensate for H⁺ loss when protonated CQ was transported out [92, 93]. Assuming that *pfvp2* functions as suggested our results indicate that in *P. falciparum* with the *pfprt* 76T genotype the *pfvp2* 405V, 582K and 711P haplotype provides the most efficient H⁺ pump.

Table 2 – The frequency of *pfvp2* alleles in *P. falciparum* with varying *pfprt* K76T and *pfmdr1* N86Y alleles

<i>pfvp2</i>	<i>Pfprt</i>		<i>Pfmdr1</i>		<i>pfprt</i> 76K		<i>pfprt</i> 76T	
	76K	76T	86N	86Y	<i>pfmdr1</i> 86N	<i>pfmdr1</i> 86Y	<i>pfmdr1</i> 86N	<i>pfmdr1</i> 86Y
405V	138/145	195/198	237/243	93/95	117/124	17/17	115/116	74/76
582K	143/146	197/198	241/244	96/97	122/125	17/17	116/116	75/76
711P	138/146	201/204	238/247	97/98	116/124	17/17	120/122	75/76
Patient samples with VKP haplotype	131/145 ^a	189/194	225/241	90/93 ^b	111/124	17/17	114/115 ^c	71/74

^a P=0.007, ^bP=0.3, ^cP=0.025. Patients with both *pfprt* 76K and 76T and patients with both *pfmdr1* 86N and 86Y were excluded. When total SNPs were compared only patients in whom all relevant alleles had been identified were included in the analyses.

Compared to all other countries the proportion of patient samples with any of *pfvp2* 405I, 582R and/or 711S was significantly more common in the following countries (table1); Liberia (P=0.01), the two West African countries Liberia + Guinea-Bissau (P=0.01), all African countries (Liberia + Guinea-Bissau + Tanzania, P=0.004) and Liberia + Guinea-Bissau + Tanzania + Honduras (P=0.01). In line with these findings there was also an association between samples with any of *pfvp2* 405I, 582R and/or 711S with *pfcr1* 76K (P=0.007) and the *pfcr1* 76K + *pfmdr1* 86N haplotype (P=0.002). Finding *pfvp2* 405I, 582R and/or 711S alleles in CQ sensitive settings in both Africa and the Americas suggests that there was a larger variation in the *pfvp2* genome prior to the spread of CQ resistance supporting the association between *pfvp2* and CQ resistance.

Fourteen of twenty patient samples with *pfvp2* 405I, 582R and/or 711S came from African countries and 11/20 from West Africa. This might suggest that the association between *pfvp2* and *pfcr1* is incidental. Though this is possible a more probable explanation for the relatively common occurrence in Africa is instead as follows: CQ resistance had not reached Liberia at the time of sampling and the proportion of CQ resistant *P. falciparum* has remained relatively low ~25% in Guinea-Bissau [147]. There has thus been less selective pressure on *pfvp2* in these 2 countries. In Tanzania the occurrence of *pfvp2* 405I, 582R and/or 711S is probably secondary to the re-emergence of the CQ sensitive *pfcr1* 76K genotype in Tanzania following the replacement of CQ with artemether + lumefantrine for the treatment of uncomplicated malaria [148].

A weakness of study II is that we only found 20 patient samples with any SNP and only 26 SNPs in *pfvp2* despite analysing 385 samples from 8 countries with varying origins and proportions of CQ resistant *P. falciparum*. Though this suggest that *pfvp2* is highly conserved it does not oppose our finding that the *pfvp2* 405V, 582K and 711P haplotype is associated with *pfcr1* 76T. Furthermore, a strength of the study is the wide time and geographic span and the fact that 3/5 chloroquine resistance origins are represented.

This is also the first report on molecular markers associated with drug resistance from Mesoamerica [149]. No SNPs associated with CQ and or SP resistance were found in Honduras indicating that *P. falciparum* remain sensitive to both drugs (Table 3). The results are in line with previous *in vitro* (1980) and *in vivo* (1998-2000) reports of CQ efficacy [150, 151]. This suggest that CQ in combination with PQ for treatment of uncomplicated *P. falciparum* malaria and SP when CQ fails as recommended in the national treatment guidelines should be efficacious [23].

Table 3 – Single nucleotide polymorphisms and amplifications in *pfert*, *pfmdr1*, *pfdhfr* and *pfdhps*

Origin	<i>pfert</i>	<i>pfmdr1</i>	<i>Pfdhfr</i>			<i>Pfdhps</i>		Number (Proportion %)	
	76	86	Copy Number	51	59	108	437		540
Honduras	K ^a	N	1	N	C	S	A	K	30 (94) ^b
Pacific	T^b	Y	1	I	R	N	A	K	1 (3)
Africa	K ^a	Y	1	I	R	N	G/A	K	1 (3)

* Resistance associated alleles are shown in bold.

^a All had the *pfert* 72-76 CVMNK haplotype.

^b Presented SVMNT haplotype. All infections were acquired in Honduras.

In Paper I we also report 2 patients with imported malaria that most probably contracted *P. falciparum* on a Pacific Island and West Africa. Both patients had ≥ 3 SNPs associated with SP resistance and the patient with *P. falciparum* from a Pacific island had the CQ resistance associated *pfert* 72-76 haplotype SVMNT (Table 1). These 2 patients highlight the risk of importing drug resistance to Honduras. La Mosquitia, from where 90% of *P. falciparum* cases of the country are reported is known to be used for drug trafficking from South America [152]. Commonly smugglers come by boat or aeroplane presumably from Colombia. It is not difficult to envisage resistant *P. falciparum* also being imported this way. The risk of resistant genes crossing over into local *P. falciparum* is thus probably highest here. Yet this has not happened. Considering this, it is worth noting that malaria is often diagnosed clinically in Honduras and that it is then treated presumptively with primaquine (0,25mg/kg) for five days in addition to CQ. Primaquine reduces the gametocytes carriage time thus reducing transmission and it is certainly possible that this has contributed to stop resistant *P. falciparum* from becoming established [153, 154]

4.2. POLYMORPHISM IN *P. VIVAX*

In paper I we report *pvmdr1* 976F allele in 7/41 (17%) samples with *P. vivax*. These results might indicate a degree of CQ tolerance but probably not resistance in Honduras [115, 155, 156]. In line with this, an *in vivo* evaluation conducted in Honduras (1998-2000) found that 73/73 *P. vivax* infections were successfully treated with CQ and primaquine [151]. We also reported 2 *pvmdr1* gene copies in one sample (with 976Y). These findings should be interpreted with caution as neither mefloquine nor artesunate are commonly used in Honduras [157]. It may however suggest the natural occurrence of this genetic change in Honduras.

Table 4 - *Pvmdr1* Y976F and F1076L haplotype proportions

Haplotypes	Y976F	F1076L	Number (Proportion %)
1	Y	F	29 (71)
2	Y	L	1 (2)
3	F	F	3 (7)
4	F	L	4 (10)
5	Y	ND	4 (10)

*Resistance associated alleles are shown in bold. ND Not Determined

Double (57L+117N), triple (57L+58R+117N) and quadruple (57L+58R+61M+117T) *pvdhfr* mutations have been associated with SP resistant *P. vivax* [158-160]. In paper I we report double mutation 57L + 58R in 2/57 (3%) samples. Allele Proportions are presented in Table 5. Similar proportions have been reported in Asia but not in South America [143, 158, 160-164]. Our results may suggest a degree of tolerance but probably not resistance to SP that should be efficacious for treatment of *P. vivax* in Honduras [76]. However, only CQ is used in Mesoamerica and efficacy of SP has not been assessed in the area [149]. Finding the double mutation despite a probably low consumption of SP suggests that resistance might develop rapidly if SP usage increases. A possible explanation for the occurrence of 57L and 58R despite the low use of SP for malaria might be that trimethoprim/sulfamethoxazole is the first line drug for treatment of acute respiratory tract infections in Honduras [165].

Table 5 – *Pvdhfr* F57L, S58R, T61M and S117N/T haplotype proportions

Alleles	F57L	S58R	T61M	S117N/T	Number (Proportion %)
1	F	S	T	S	57 (97)
2	L	R	T	S	2 (3)

* Resistance associated alleles are shown in bold

5. CONCLUSIONS

Paper I

In *P. falciparum* infections originating in Honduras only SNPs linked to chloroquine or sulphadoxine-pyrimethamine sensitivity were found indicating that chloroquine and sulphadoxine-pyrimethamine should remain efficacious.

Chloroquine and sulphadoxine-pyrimethamine resistance associated SNPs were found in patients that contracted *P. falciparum* overseas highlighting the risk of drug resistance being imported to and spreading in Honduras.

In *P. vivax* infections contracted in Honduras genetic polymorphisms associated with chloroquine and sulphadoxine-pyrimethamine tolerance were found in eight (13%) and two (3%) samples, respectively, suggesting that a degree of tolerance exists in the country.

Paper II

There was a significant association between the chloroquine resistance causing *pfcr* 76T and the *pfvp2* haplotype 405V, 582K and 711P. This indicates that there is a specific *pfvp2* haplotype that is associated with chloroquine resistance. The results are in line with previous data indicating that *pfvp2* is involved in chloroquine resistance. However, the number of samples with SNPs in *pfvp2* was small.

6. FUTURE

We are currently assessing *pfvp2* amplification to complete study II. A detailed sequencing based study to describe parts of the genome in resistance associated *pfmdr1*, *pfdhfr*, *pfdhps*, *pfhhe1* (Sodium/Hydrogen exchanger gene 1) and possibly other genes in Liberian samples is planned. We are also planning an *in vitro* study to determine if *P. falciparum* are able to “shut down” when stressed. When both CQ sensitive and resistant *P. falciparum* are exposed to high chloroquine concentrations for periods of up to one week very few small dots generally considered to be dead parasites remain. When these dots are followed for a long enough period of time they often revive. A hypothesis to explain this finding is that *P. falciparum* have an innate ability to shut down all non essential systems and thereby survive. This could in turn explain why *P. falciparum* survive treatment even when adequate concentrations of a drug to which they are not resistant are achieved. Similar mechanisms have been described in bacteria and are believed to be a survival response to for example lack of essential nutrients.

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