Universidade do Minho
Escola de Medicina

Isaac Miguel Sánchez

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Doutora Maria Isabel Mendes Veiga
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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

The work presented in this thesis was performed in the Microbiology and Infection Research Domain in the Life and Health Sciences Research Institute (ICVS), University of Minho, Braga, Portugal and Institute of Microbiology and Parasitology (IMPA) from Autonomous University of Santo Domingo (UASD), Dominican Republic. Financial support was provided by the FEDER funds through the Operational Programme Competitiveness Factors – COMPETE and Portuguese National funds through FCT – Foundation of Science and Technology under the project POCI-01-0145-FEDER-007038 and by the project NORTE-01-0145-FEDER-000013, supported by Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, through the European Regional Development Fund (ERDF), and by the Dominican Republic National Fund for Innovation and Development of Science and Technology, Ministry of Higher Education Science and Technology (grant numbers 2013-2A2-002 and 2014-2A2-073).

Abstract

Malaria is still a disease of major concern globally. In 2017, the malaria incidence was of 219 million cases and 435,000 people died because of it. Five parasites species of the genus Plasmodium cause the ailment in humans. Out of these, *P. falciparum* and *P. vivax* are responsible for most cases. Dominican Republic (DR) is a malaria endemic country that is now under the elimination phase. In DR, as malaria prevalence declines, the proportion of cases that fall below the detection level of standard malaria diagnosis (rapid diagnostic tests (RDTs) or microscopy) might be increasing, underlining the need of including more sensitive diagnostic methods such as molecular tools like PCR and LAMP. Furthermore, DR is one of the few countries in the world where chloroquine together with primaquine is used as the first-line treatment for uncomplicated *P. falciparum* malaria. This treatment is still clinically effective in DR, even though the world is struggling with resistance to newer antimalarials. The aim of this thesis is to understand the influence of host-pathogen genetic variants on malaria chemotherapy with emphasis on the DR situation. We have genotyped the *Glucose-6-Phosphate Dehydrogenase A-* allele, a predictor of possible hemolytic anemia due primaquine treatment, in Dominican subjects and found a high prevalence of this allele circulating in DR. Nevertheless, there is no side effects reported in DR upon primaquine treatment concluding that the dose used in DR is safe even in subjects with *Glucose-6-Phosphate Dehydrogenase A-* allele. In the same subset of samples, that through malaria standard diagnostic tests did not detect the malaria parasite, we explored for malaria positivity through molecular genotyping. We detected a high percentage (20%) of false negatives highlignting the need to change to more sensitive diagnostics tests in the country. We genotyped the false negative for chloroquine resistance markers and detected mutations on the *pfcrt* and *pfmdr1* genes. This is the first report showing the presence of this mutations in the country. In this thesis work we have also attempt to gene edit, through selection linked integration (SLI) system the *pfcrt* to allelic exchange the mutation found and to exchange *pfmdr1* on *P. falciparum* 3D7 strain by its orthologue *pvmdr1* as a proof of concept that it is possible to use *P. falciparum* as a vehicle to study *P. vivax* genes.

Resumo

A malária ainda é uma doença de grande preocupação global. Em 2017, a incidência da malária foi de 219 milhões de casos e 435.000 pessoas morreram devido à mesma. Cinco parasitas do género Plasmodium causam doença em humanos. Destes, *P. falciparum* seguido de *P. vivax* são responsáveis pela maioria dos casos. A República Dominicana (RD) é um país endémico da malária que está agora em fase de eliminação. Na RD, a prevalência da malária tem vindo a decrescer, aumentando a proporção de casos abaixo do nível de detecção do diagnóstico padrão da malária (testes de diagnóstico rápido (TDR) ou microscopia), alertando para a necessidade de métodos diagnósticos mais sensíveis como ferramentas moleculares (PCR e LAMP). Além disso, a RD é um dos poucos países do mundo onde a cloroquina juntamente com a primaquina constituem o tratamento de primeira linha para malária não complicada por *P. falciparum*. Na DR este tratamento ainda é clinicamente eficaz, embora o resto do mundo tenha problemas deresistência a antimaláricos ainda mais actuais. O objetivo desta tese é compreender a influência das variantes genéticas do patógeno-hospedeiro na quimioterapia da malária, com ênfase na situação da RD. Para avaliar o risco de anemia hemolítica devido a tratamento com primaquina, analisámos a prevalência da deficiência de glicose-6-fosfato desidrogenase em indivíduos Dominicanos através de genotipagem do alelo *G6PD A-*, tendo-se observado uma elevada prevalência deste alelo. Contudo, não existem efeitos secundários reportados na RD após tratamento com primaquina, pelo que a dose usada é segura, mesmo em sujeitos portadores de *G6PD A-.* No mesmo subconjunto e para amostras onde os testes de diagnóstico padrão não detectaram o parasita da malária, estas foram testada para positividade de malária através de genotipagem molecular. Detectando cerca de 20% de positividade para *P. falciparum* neste conjunto de amostras, analisamos os marcadores de resistência à cloroquina nos genes *pfmdr1* e *pfcrt*. Na tentativa de entender melhor a interação entre os genes *pfcrt* e *pfmdr1* e o impacto de diferentes variantes na resistência aos antimaláricos, estirpes adaptadas em laboratório foram geneticamente editadas usando uma ferramenta de edição do genoma recentemente descrita, denominada integração ligada à seleção (SLI). Finalmente, para melhor compreender os mecanismos de resistência a drogas em *P. vivax*, dado que esta espécie de *Plasmodium* provou ser difícil crescer *in vitro*, tentámos substituir o *pfmdr1* em *P. falciparum* 3D7 pelo seu ortólogo *pvmdr1,* usando SLI como prova de conceito de que é possível usar o *P. falciparum* como veículo para o estudo dos genes de *P. vivax*.

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List of abbreviations and acronym

ABC – ATP-binding cassette

ACT – Artemisinin-based combination therapy

AL – Artemether-lumefantrine

AQ – Amodiaquine

ART – Artemisinin

AS-AQ – Artesunate-amodiaquine

AS-MQ – Artesunate-mefloquine

AS-SP – Artesunate-sulfadoxine-pyrimethamine

ATM – Artemether

bsd – Blasticidin S deaminase gene

BSD – Blasticidin S HCl

cDNA – Complementary DNA

CQ – Chloroquine

G6PD- glucose-6-phosphate dehydrogenase

hDHFR – Human Dihydrofolate reductase

DV – Digestive vacuole

hDHFR – Human dihydrofolate reductase

hMRP – Human multidrug resistance protein

IC50 – Half maximal inhibitory concentration

LB – Luria-Bertani medium

MCM – Malaria culture medium

PBS – Phosphate-buffered saline

PCR – Polymerase chain reaction

PfCRT – *P. falciparum* chloroquine resistance transporter

PfHRP2- *P. falciparum* Histidine rich protein 2

PfMDR1 – *P. falciparum* multidrug resistance 1

PMQ – Primaquine

PvMDR1– *P. vivax* multidrug resistance 1

rpm – Rotations per minute

SLI- selection linked integration

SNP – Single nucleotide polymorphism

SOB – Super optimal broth

WHO – World Health Organization

1. Introduction

1.1 Malaria epidemiology

1.1.1 Global Burden

The latest data from the World Health Organization (WHO) shows that despite the trend in global reduction of malaria cases, between 2015 and 2017 no significant progress in reducing malaria has been made. In fact, in 2017 we saw 3 million more cases compared with 2016.. Despite a 18% reduction in the global malaria incidence between 2010 and 2017, substantial increases in case incidence occurred in the Region of the Americas, primordially in Brazil, Nicaragua and Venezuela. According to the 2018 world malaria report, 219 million cases of malaria occurred in 2017 worldwide with 93% occurrence in Africa. That same year there were an estimated 435 000 deaths the majority (266 000) of which were children under 5 years of age [1][2].

In sub-Saharan Africa *P. falciparum* is the most prevalent malaria parasite, accounting for 99,7% of estimated malaria cases in 2017. Outside of the African Continent *Plasmodium vivax* is the predominant parasite accounting 74.1% of cases[1].

1.1.2. Malaria in the Dominican Republic

Dominican Republic (DR) is an endemic. malaria country. The population at risk of contracting was estimated to be of 5 930 679 in 2017, nearly half of the Dominican population. In DR effective control measures for prevention and diagnosis has resulted in a 50-75% reduction of the disease burden over the last decade. According to the 2018 Malaria World report, in 2017 there were 341 reported cases and no death, with all cases reported that year caused by *Plasmodium falciparum*[1]. However, from January 2018 to November 2018, 34(8%) cases of imported *P. vivax* mainly from Venezuela as reported by the epidemiology department of the Dominican ministry of health[3].

Recognizing the decrease of the malaria burden in the DR in the last decade, DR is now under a strategic plan towards malaria elimination with support of the Pan American Health Organization (PAHO/WHO), the Global Fund to fight malaria, the United Nations Sustainable Development Goals and other partners [4].

To achieve elimination, additional measures should be taken to complement the already successful strategies that the country has implemented including more sensitive diagnostic methods such as molecular tools like polymerase chain reaction (PCR) and loop mediated isothermal amplification (LAMP). Furthermore, DR is one of the few countries in the world where chloroquine, used as first-line treatment for uncomplicated *P. falciparum* malaria, is still clinically effective. Yet, up to date, there is limited information on the molecular characteristics of the malaria parasite circulating in DR [5]. Knowing that in the neighboring country (Haiti), chloroquine is no longer effective[1], highlights the importance of monitoring the presence in the DR parasites, of molecular markers of anti-malarial drug resistance.

1.2 The malaria parasite

1.2.1 *Plasmodium* biology

Malaria *Plasmodium a*re classified as belonging to the phylum *Apicomplexa.* The biology of these parasites require, to complete their life cycle, two hosts. The exogenous sexual phase (sporogony) of the *Plasmodium* life cycle occurs in several species of *Anopheles* mosquitoes and the asexual phase (schizogony) occur in the vertebrate host (Reptiles, birds and mammals). More than 200 species of *Plasmodium* have been described [6]. To date five Plasmodium species are known to infect humans: *Plasmodium falciparum, P. knowlesi P. malariae, P. ovale and P. vivax.* After the inoculation by mosquito, sporozoites undergo development and multiplication in the parenchymal cells of the host liver, followed by a blood phase, shown in the section below, that is ultimately responsible of the appearance of clinical manifestations [7]. The main differences between human malaria parasites are shown in table 1.

1.2.2. The red blood cell cycle

In 1976 the first article describing the continuous culture of *Plasmodium falciparum* in human erythrocytes was published. With this work William Trager was able to observe all stages of the erythrocytic cycle of the parasite [8]. Since then, our understanding of the stages of the *Plasmodium* asexual cycle has increased and continues to do so. The pathogenic red blood stages of malaria parasites are also the target to which most antimalarials have been designed to act on [9], [10].

Once merozoites have invaded erythrocytes, they mature over the course of 24-72 hours depending on the species[7] (table 1). During maturation parasites go through three distinct morphological stages[11]. First is the 'ring stage'. It is believed that host cell modifications are a major task of this stage. The second

phase, the 'trophozoite stage', is characterized by rapid parasite growth and the appearance of haemozoin in the food vacuole (FV) [12]. The final stage is the production of bloodschizonts each one containing 6 to 36 merozoites, followed by erythrocytes rupture and the release into the blood of new merozoites that will invade new red blood cells (RBC). In the blood some intraerythrocytic stages differentiate into gametocytes. After the female *Anopheles* mosquito feeds on the blood of a plasmodium infected host the gametocytes are responsible for the sporogonic cycle into the mosquito midgut [12][13].

Table 1 : Characteristics of malaria infection from the five species of *Plasmodium* infecting humans. Source: [7]

Characteristics	*P. falciparum*	*P. knowlesi*	*P. malariae*	*P. ovale*	*P. vivax*
Liver stage (days)	5-7	8-9	14-16	9	6-8
Erythrocytic cycle (Hours)	48	24	72	50	48
Red cells affected	All	All	Mature erythrocytes	Reticulocytes	Reticulocytes
Severe malaria	Yes	Yes	No	No	Yes
Relapses from liver forms (hypnozoites)	No	No	No	Yes	Yes
Stages sequestered away from circulation	Inmature gametocytes (stages I-IV)	-	-	-	-
Stages seen in peripheral blood	Mature gametocytes stage V.; young rings	All blood stages	All blood stages	All blood stages	All blood stages

1.2.3 *Plasmodium* genome

Plasmodium falciparum whole genome was first described by Malcolm J. Gardner in 2002. The *P. falciparum* 3D7 nuclear genome is composed of close to 23 mega base pairs (Mbp) distributed among 14 chromosomes. *P. falciparum* genome is very AT-rich composition of 80.6% and rises to 90% in introns and intergenic regions. About 5,300 protein-encoding genes were predicted, estimating an average gene density of 1 gene per 4,338 base pairs (bp) [14]. Today large dataset of genomic data on plasmodium falciparum are available on open access. The "MalariaGEN pf3k project" has released genome sequence data from close to 3000 samples and the "*Plasmodium* Community project" has publicly available the

data on the analysis of more than 7000 samples from around the globe [15],[16]. Recently a functional profiling of *Plasmodium* genes was done using *P. berghei* and determined that it requires two-thirds of genes for optimal growth. They also found, extreme functional redundancy among expanded gene families operating at the parasite-host interface [17]. Transcriptomic analysis of *P. falciparum* intraerythrocytic developmental cycle have revealed that almost every gene is expressed during this stage. However, 75% of the genes are only activated once during this cycle, illustrating a close association between transcriptional regulation and the developmental progression [18]. However a recent study using single cell RNA sequencing suggests that expression over development is not as continuous as commonly thought.[19]

There are practical obstacles to genome sequencing of *P. vivax*. Continuous culture of the parasite has proven difficult and clinical samples are low levels of parasitemia and can only be cultured for a few days, therefore getting sufficient amount of genetic material is a challenge[20]. To date, molecular studies of *P. vivax* have relied on the Salvador-I reference genome sequence, derived from a monkey-adapted strain from South America. However, the Salvador-I reference remains highly fragmented [21]. There are now genomic databases for *Plasmodium vivax*. Currently, the MalariaGEN "*P. vivax* genome variation project" has released sample and genotype information for 228 samples from 13 countries [22]. So far 12–14 linear chromosomes that range in size from 1.2 to 3.5 Mbp have been identified in *P. vivax*. Initial estimates put the genome size at 35–40 Mb. The *P. vivax* nuclear genome exhibit a marked bias towards AT nucleotide composition. The chromosome ends appear to consist of AT-rich sequences, while telomere-distal regions seem to be GC-rich sequences. By contrast, *P. falciparum* has an even GC distribution along the genome [23].

1.3 Malaria diagnosis

Critical for malaria control and elimination is an accurate diagnosis. Malaria diagnosis is done mainly by three techniques: blood smear microscopy, malaria rapid diagnostic tests (RDTs) and molecular diagnosis such as polymerase chain reaction (PCR)[24].

Conventional laboratory diagnostic of malaria is done by microscopic examination of stained blood films using Giemsa, Wright's, or Field's stains [25]. Due to its simplicity, low cost, its ability to identify the presence of parasites, the infecting species, and assess parasite density, it is still widely used. However, microscopy requires sizable expertise and trained technicians with average microscopist detection of 50-100 parasites/µl [26].

Since their development in the early 1990's Malaria rapid diagnostic test (RDT) have become widely used, especially in field studies, where access to a laboratory with the capacity to perform microscopic analysis is difficult [24]. Rapid diagnostic tests use chromatographic strips to detect parasite antigens including histidine-rich protein-2 (HRP-2) or lactate dehydrogenase (pLDH)[27]. Although less specific than the pLDH detection test, the HRP-2 test is preferred to detect uncomplicated malaria, as its sensitivity is comparable to that of microscopy. Yet, reports showed that some parasites carry deletions of the antigen, resulting in false negative diagnosis [27]. Another shortcoming of RDTs, is that just like microscopy, detection limit goes up to 100 parasites/μl [28]. (To evaluate RDTs performance at low parasite density limit used is parasites sample are diluted to 200 parasites/μl as it is below the mean parasite density found in many malaria endemic areas [29]) Rapid Diagnostic tests is used in conjunction with other methods to confirm the results, characterize infection, and monitor treatment [28].

Molecular diagnostics based on nucleic acid amplification-based tests (NAATs), such as polymerase chain reaction (PCR) have higher sensitivity (0.5 to 10 parasites/μl) [30] than that of microscopy or RDTs. Not yet common as routine diagnostic, PCR is increasingly being used for confirmatory diagnosis of returning travelers or migrants suspected of malaria in developed countries and in some reference laboratories in endemic areas. However PCR requires well equipped laboratories, expensive supplies and trained technicians, implementing this technologies in developing countries remains a challenge [31]. However, a new NAAT tool, loop-mediated isothermal amplification method (LAMP), that is easier to use and requires less equipment than PCR, is a promising candidate to be used as a diagnostic tool in field studies and in regional clinics [32].

1.4 Malaria control and elimination strategies

The WHO Global technical strategy for malaria 2016-2030, cites four goals for this period: reduce malaria mortality rates globally compared with 2015, reduce malaria case incidence globally compared with 2015, eliminate malaria from countries in which malaria was transmitted in 2015 and prevent re-establishment of malaria in all countries that are malaria-free[33].

To reach these goals several challenges needs to be overcomed. For one, many people infected with malaria parasites remain asymptomatic or undiagnosed. Moreover, there are settings were parasitemia is very low in a considerable number of individuals and cannot be detected with current routine diagnostic tools. These people constitute an infectious parasite reservoir and contribute to the cycle of malaria

transmission. The other challenges being the emergence of drug resistance and in countries where both *P. falciparum* and *P. vivax* are present, reducing the burden due to *vivax* is harder due to hypnozoites being undetectable and leading to relapses [33].

The most recent guidelines for eliminating malaria include 4 components. Enhancing and optimizing vector control and case management, increasing the sensitivity and specificity of surveillance to detect, characterize and monitor all cases (individual and in foci), accelerating transmission reduction and investigating and clearing individual cases, managing foci and following up [34]. Out of these, the characterization of cases is of the outmost importance given that drug resistance remains a serious threat. Knowing the prevalence of drug resistant parasites in a given population as well as understanding the mechanisms of antimalarial drug resistance and action is key to achieve elimination.

1.5 Malaria chemotherapy

1.5.1 Global overview

Based on the mode of action, the antimalarial drugs are mainly classified into three groups: quinolones, antifolates and artemisinin derivatives [35]. These drugs main targets are the erythrocytic stages of malaria parasites, responsible for symptomatic human illness. There are differences in the drug regiments used to target the two most prevalent malaria parasites, *P. falciparum* and *P. vivax* [36]. For uncomplicated *P. falciparum* malaria, the WHO recommends, for most countries where malaria is endemic, the use of artemisinin-based combination therapy (ACT). ACT is a treatment with two effective antimalarials with different chemical structure, one with a short half-life acting, artemisinin derivative, combined with a long half-life acting antimalarial [37]. It can be presented with different combinations denoted in Table 2. The combination goals are to improve treatment efficacy and delay the development of resistance.

To reduce transmissibility, WHO recommends the use of a single dose of primaquine (0.25 mg/kg), a gametocidal drug. Primaquine is also effective at the liver stages of the parasite and as so, used to treat *P. vivax* hypnozoites, to avoid relapses [table 1].

Table 2. WHO recommended artemisinin-based combination therapies.

Short acting artemisinin derivative	Combination Drug
Artemether	Lumefantrine
Artesunate	Amodiaquine
Artesunate	Mefloquine
Dihydroartemisinin	Pipiraquine
Artesunate	Sulfadoxine-pyrimethamine
***Artesunate**	Pyronaridine

** It is the only ACT to be granted a positive scientific opinion under the European Medicines Agency's (EMA) Article 58 procedure, and is the only ACT to be specifically indicated for the blood-stage treatment of the two main strains of malaria: P. falciparum and P. vivax. WHO currently recommends artesunate-pyronaridine in areas where other ACTs are failing. Source:*[37]

1.5.2 Malaria chemotherapy in the Dominican Republic

In DR the recommended first-line treatment for uncomplicated *Plasmodium falciparum* malaria is chloroquine (CQ)(25 mg/Kg) split into three daily doses administered together with single dose of primaquine (PQ) as a gametocidal (0.75 mg/Kg) [38]. For *P. vivax* first line treatment follows the WHO recommendation treating with a combination of CQ (25 mg/Kg) plus PQ (0.25-0.5 mg/Kg) for 14 days combination.

Chloroquine has been shown to be clinically effective with no report up to today of its clinical failure. Nevertheless, in 2016 forty courses of Artemether-lumefantrine, the recommended second line treatment were delivered [39].

1.5.2.3 Chloroquine

Chloroquine is a 4-Aminoquinolone and like other drugs of this family is a blood schizontocides. After the end of World War II, this synthetic drug became the drug of choice for treatment of uncomplicated malaria and was used by WHO in the malaria eradication campaign launched in 1955. Due to the spread of CQ resistance, its use in the treatment of uncomplicated *P. falciparum,* has been discontinued in most countries, it remains the first line treatment in limited areas of Central America and the Caribbean region (*i.e.* Dominican Republic), where it retains its efficacy [39],[40]. Even though CQ resistant *P. vivax* infections have been observed, CQ is still the primary drug of choice for *P. vivax* in most parts of the world.

1.5.2.4 Primaquine

Primaquine, on the other hand, is used in the case of *P. falciparum* infections to target gametocytes and stop transmission in a single dose. This is a drug that must be carefully administered given the fact that it can be a trigger for hemolytic anemia in patients that are susceptible to oxidative stress such as those that carry mutations that confer glucose-6-phosphate dehydrogenase deficiency [41]. Primaquine is so far the only recommended drug to target hypnozoites in *P. vivax* infected patients and it is administered daily for 14 days [42].

1.5.2.4.1 Risk of primaquine in patients with G6PD deficiency

Geographical distribution of hemoglobinopathy genetic traits, such as the glucose-6-phosphate dehydrogenase (G6PD) deficiency, is long known to correlate with reduced severity of malaria [43]. The high prevalence of this enzymatic defect in African populations corroborate with this correlation [44]. G6PD deficiency interferes with the enzymes role in providing a reductive shield to the RBC through a supply of NADPH. As a consequence of that it can lead to hemolytic anemia upon exposure to oxidative medication, with severity dependent on the dose of the drug and the degree of deficiency [45]. G6PD deficiency is inherited as an X-linked trait, with more than 300 mutant alleles described [46]. *G6PD A-* is the most common variant in African population that can lead to hemolytic anemia in patients exposed to the antimalarial primaquine [47]. Indeed, G6PD deficiency was discovered in the 1950s following reports of primaquine-induced haemolysis. [48]

To diminish the risk of haemolysis, in mass drug administration programs, the reinforcement of artemisinin based combination therapies (ACTs) with PQ is done in a single low dose (0.25mg/Kg), as recommended by the WHO [49]. Yet, recent publication have showed that the concentration of the single dose of primaquine can be increased without causing significant hemolysis in G6PD deficient patients[49],[50],[51].

1.5.3 Antimalarial drug resistance

1.5.3.1 Emergence of antimalarial drug resistance

First developed in the 1930s, chloroquine, became the most widely used synthetic antimalarial during the 1960s and 1970s. Foci of resistant *P. falciparum* were detected in Colombia and at the Cambodia-Thailand

border during the late 1950s, and by the late 1970s, chloroquine resistance was widely spread around the globe [52].

The rise in chloroquine resistance contributed to a worldwide increase in malaria-related mortality, particularly in sub-Saharan Africa. Alternative synthetic drugs such as sulfadoxine–pyrimethamine and mefloquine were later developed; resistance to theses quickly emerged as well [52]. To avoid emergence of resistance a new therapy artemisinin, a very fast acting drug, in combination with a long half-life drug (ACT)- started to be widely used only in uncomplicated malaria cases. After this restriction was lifted in 2009, parasites with reduce sensitivity to ACTs have been detected in multiple locations in South East Asia. [52]

Emergence of antimalarial drug resistance seems to be inevitable. Until new drugs become available, the molecular mechanism of drug action and resistance must be understood. To help in the development of new treatments and identifying new molecular markers of anti-malarial drug resistance in *Plasmodium falciparum*. This knowledge is of great help when deciding what drugs to use in the various epidemiological contexts.

1.5.3.2 Mechanisms of chloroquine resistance

Chloroquine (CQ) acts by interfering with the sequestration of toxic heme that is produced when hemoglobin is digested by the intra-erythrocytic parasite. In the acidic environment of digestive vacuole hemoglobin is digested forming the heme. The heme detoxification process then follows by its oxidation into hematin which in turn is bio-crystallized into the non-toxic malaria pigment Hemozoin [53]. Chloroquine binds to heme, preventing the detoxification process and producing complexes that are detrimental to both membranes and enzymes. These are ultimately lethal to the parasite [40],[54]. Chloroquine resistance in *Plasmodium falciparum* has been widely studied using different approaches.

Polymorphisms in genes coding for two transporter proteins have been linked to *P. falciparum* resistance to CQ. The chloroquine resistance transporter gene *pfcrt* and the multi drug resistance transporter gene *pfmdr1*. Both proteins are located in the membrane of the food vacuole and are essential for the traffic of solutes to and from the cellular compartment [55].More details about the mechanisms of CQ resistance will be explained in the following sections.

1.6 Resistance associated genes

1.6.1 *Plasmodium falciparum chloroquine resistance transporter* (*pfcrt*)

The PfCRT is a member of the drug/metabolite transporter superfamily of electrochemical potential-driven transporters [56]. Mutations in amino acid positions 72-76 have been linked to chloroquine resistance. The K76T mutation is determinant in conferring resistance to chloroquine.[57] Drug accumulation studies comparing parasites carrying wild type and mutant alleles of *pfcrt* have shown that in mutant parasites, a lot less chloroquine is accumulated inside the food vacuole, pointing to PfCRT has being an efflux pump.[58] The *pfcrt* K76T mutation increases the transporter protein's affinity to chloroquine, therefore moving the drug away from its target and conferring resistance [56],[59],[60].

Molecular epidemiological and in vitro studies with common ACT partner drugs have shown that lumefantrine (arylaminoalcohol related to mefloquine), selects for wild type *pfcrt*. By contrast, the amodiaquine partner appears to select for mutant forms of *pfcrt* in field isolates [56][61][62]

1.6.2 *Plasmodium falciparum multidrug resistance 1* (*pfmdr1*)

In the 90s when first reports about chloroquine resistance appeared. The first candidate was the *pfmdr1*. When performing phenotypical studies of chloroquine resistant (CQR) parasites it was found that the calcium agonist verapamil reversed the expulsion of the drug from the cell while reversing the resistant phenotype. This observation compared to similar studies performed in mammalian tumor cells with a multidrug resistant phenotype (MDR) led to believe that chloroquine resistance in *Plasmodium falciparum* and drug resistance in tumor cells shared a similar mechanism [63].

It was also observed that PfMDR1 the homolog in *P. falciparum* to the human P- glycoprotein had gene duplication in CQR parasites [64]. Only after, using a genetic cross, resistance to CQ was mapped to chromosome 7 [65]. The first gene candidate as a determinant of CQR was *cg2* [66] later confirmed to be the *pfcrt* [57] with K76T being the most responsible [57],[67],[68].

PfMDR1 is an ATP-binding cassette transporter and a homologue of the human multidrug-resistance-conferring P-glycoprotein [55]. It is also imbedded in the membrane of *Plasmodium falciparum* food vacuole. Point mutations on the *pfmdr1* gene have been associated with reduce sensitivity to chloroquine in the context of mutant *pfcrt.* PfMDR1 is an influx pump into the parasite food vacuole, and mutations on the gene are thought to reduce the amount of chloroquine that can enter the food vacuole. Five globally

prevalent amino acid substitutions have been identified. Two amino-terminal mutations, N86Y and Y184F, and three carboxy-terminal mutations, S1034C, N1042D and D1246Y [55].

The ability of PfMDR1 variants to influence antimalarial drug potency is supported by earlier transfection studies delineating the role of the C-terminal *pfmdr1* mutations [69]. Recent work from our group has also elucidated the role of N-terminal *pfmdr1* mutations [55]. This type of genome editing studies allow to deplete the complexities intrinsic to field–based studies, including contributions of polyclonal infections and host immunity, revealing the allele variance influence in antimalarial response. The role of PfMDR1 in CQR was confirmed using a genetic cross between two resistant CQ strains (7G8xGB4). PfMDR1 was shown to influence the degree of resistance in the CQ resistant progeny of the Dd2xHb3 cross [70],[71].

1.6.3. The interplay of PfCRT and PfMDR1 in resistance

Plasmodium falciparum chloroquine transporter (PfCRT) and *Plasmodium falciparum* multi drug-resistance 1 transporter (PfMDR1), both reside on the membrane of the parasite's food vacuole (DV), where they are thought to regulate the flux of solutes across this membrane [55], [72]. Because PfMDR1 is an influx pump and PfCRT an efflux pump, point mutations in the genes encoding for these transporters can alter their affinity to different antimalarial drugs. Different combinations of mutant *pfcrt* and *pfmdr1* are believed to play a role in whether drugs are moved to the cytoplasm or the food vacuole. Quinolones and artemisinin derivates interact with the heam/hemozoin metabolism inside the food vacuole. Mefloquine an arylaminoalcohol shows increased succptibility mediated by mutant pfcrt yet lumefantrine a related drug selects for wild type *pfcrt* [56][73]. Depending the compartment where the target of drug in question is located, variations in both these genes can play a very important role in modifying the susceptibility / resistance profile malaria parasites [55],[69],[72].

Using the same genetic crosses described by Sá *et al.*, lower level of CQR and higher monodesethylamodiaquine resistance (MDAQR) in the progeny harboring the south American *pfcrt* allele was demonstrated to be the result of contributions from both *pfcrt* and *pfmdr1* alleles [70].

1.7 Selection linked integration system to study Plasmodium genes

7.1 Overview

Genetically modification of essential genes in *Plasmodium falciparum* require integration of episomal plasmids, which is an inefficient and time intensive process. In order to overcome this a new system termed "selection linked integration" was developed by Jakob Birnbaum *et al.* [74].

Briefly, a promoter-less targeting region on the plasmid to be integrated is linked with an additional selectable marker that is separated by a skip peptide. Only after single crossover integration into the target locus, the selectable marker can be expressed, the skip peptide allows the target and the marker to be translated together into separate proteins. Parasites carrying the integration can be selected by using the additional integration-linked resistance marker [figure 1], [74].

Here we adapted the system to use it as a gene replacement tool instead of the functional protein analysis tool presented by the original authors.

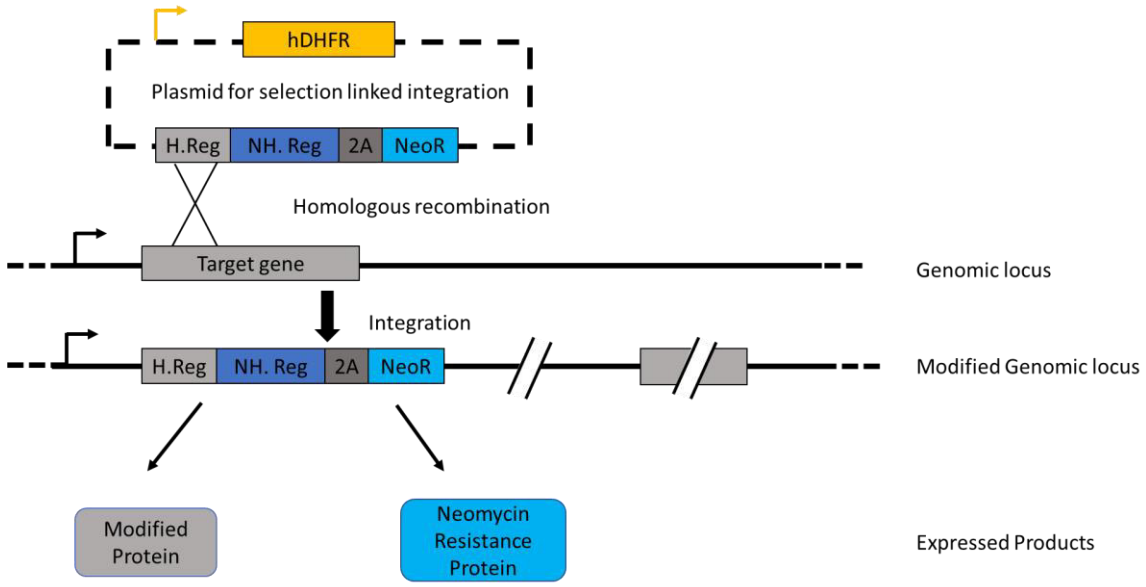

Figure 1: Selection linked integration. Arrow: promoter; H.Reg: Homologous region; NH. Reg: Non- Homologous region; 2A: **T**2A skip peptide; NeoR: neomycin resistance gene (Integration selection marker); hDHFR human dihydrofolate reductase (plasmid selection marker). modified from [74]

1.7.1.1 In-vitro drug resistance studies in *Plasmodium falciparum*

Continuous culture of *Plasmodium falciparum* has been possible since the 1970 [8]. Together with genome editing tools and *in-vitro* drug susceptibility tests, has enabled *in-vitro* studies, it has contributed to the elucidation of the molecular mechanisms of antimalarial drug action and resistance.

The introduction of exogenous DNA into *P. falciparum* requires the passage through several membranes. In the 1990s, transfection technologies allowed for considerable development of *P. falciparum* genomic analysis [75]. Different transfections techniques were tried, the most reliable and still used today is the electroporation, described by Yimin Wu *et al.* in 1995 and improved by David Fidock and Thomas Wellems in 1997 [75], [76], [77].

The first tries at introducing exogenous DNA into *Plasmodium falciparum* only allowed for transient expression of chloramphenicol acetyltransferase (*CAT*) gene [76]. The integration of plasmids into the genome is essential to allow the study of *P. falciparum* genes. Even though advances in this area have been made, in part due to the development of positive and negative selection markers[77], [78], [79], [80]. The process is still inefficient mainly due to the rarity of homologous recombination event in *P. falciparum* [74].

Recent advances in genome editing tools such as Zinc-Finger Nucleases and the CRISPR/Cas9 system, allowing precise gene editing by triggering a specific double-stranded break adjacent to the targeted single nucleotide polymorphism (SNP) with DNA lesion repair promoted by homology-directed recombination, has been successfully used in *P. falciparum* and have been significant to understand the role of point mutations in drug resistance [55], [80], [81].

1.7.1.2 *In-vitro* drug resistance studies in *Plasmodium vivax*

In order to find molecular drug-resistant markers in *P. vivax* researchers have relied on the known molecular markers in *P. falciparum* [82]. The Y976F mutation in *Plasmodium vivax* multidrug resistance drug gene 1 (*Pvmdr1)*, an orthologue to *Pfmdr1,* as well as increased copy number, have been associated with increased sensitivity to chloroquine *in vivo* [83],[84],[85]. Unlike in *P. falciparum* where mutations in *Pfcrt* have been linked to CQ resistance, mutations in the *P. vivax* orthologue *Pvcrt* have not been linked to field CQ resistance [86]. Furthermore, because *P. vivax* prefers to grow inside reticulocytes, long term culture of this species has been proven difficult, making it almost impossible to employ similar strategies as in *P. falciparum* (*i.e.* genetic modifications and in vitro studies)[35].

2. Aims

The main goal of this work is to understand the host-pathogen genetic variants impact on malaria drug response with emphasis on Dominican Republic context. The specific aims of the studies presented in this dissertation are:

Aim 1: Evaluate the prevalence of G6PD genetic variants in Dominican Republic

Given the fact that primaquine, an antimalarial drug with hemolytic effects, is used in DR we explore the prevalence of the G6PD deficient allele, *G6PD A-* (containing the pathogenic G202A substitution) in Dominican subjects.

Aim 2: Detect the prevalence of low-density malaria infections in Dominican Republic

Under a strategic plan towards malaria elimination in Dominican Republic, standard diagnosis such as microscopy or antigen recognition, fails at detecting parasitemias lower than 50 parasites/μL of blood. Clearing this parasite reservoir is critical for malaria elimination, as these individuals remain infectious to the mosquito, promoting the disease transmission.

Here, through molecular methods we aim at:

a. Determine the proportion of false-negative samples diagnosed through RDTs or microscopy.
b. Determine the Plasmodium species present in the false-negatives samples
c. Determine the prevalence of *pfhrp2* deletions in the false-negative samples, to understand the cause of false-negatives in the case of the samples diagnosed with RDTs.
d.

Aim 3: Determine the presence of chloroquine molecular markers of resistance in Dominican parasites, namely the PfCRT K76T and the PfMDR1 N86Y single nucleotide polymorphism.

Aim 4: Explore the impact of resistance factors in antimalarial response

a- Understand the interplay of the food vacuole transporters, PfCRT and PfMDR1 by:
    a. Using verapamil, a partial mixed-type inhibitor of chloroquine transport via PfCRT
    b. By allelic exchange PfCRT at aminoacid positions 72-76.

b- The generation of a hybrid Pf/PvMDR1 as a concept to study *P. vivax* resistance genes using *P. falciparum* as a model.

3. Material and Methods

3.1 Study 1: Prevalence of G6PD genetic variants in Dominican Republic

3.1.1. Study setting, participants and ethics:

A total of 331 febrile patients suspected of having malaria, that when diagnosed either by light microscopy (94) or by First Response® Malaria Ag. *P. falciparum* (HRP2) Card Test (237) results were negatives. Samples were collected at the Jaime Mota Regional Hospital in Barahona (245), Vinicio Calventi Hospital (3) ,Robert Reid Cabral Hospital (1) in Santo Domingo and the San José de Ocoa Hospital (1), as well as in primary health care centers in Barahona (73), Santo Domingo (4), La Altagracia (2), Bahoruco (1) and La Vega (1) provinces [Figure 2].

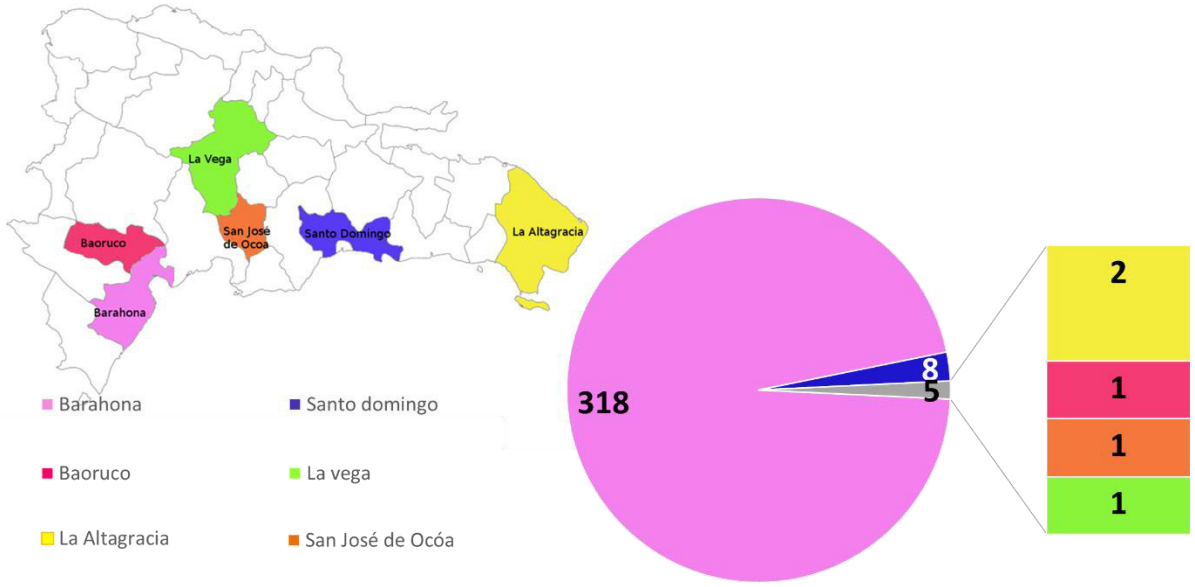

Figure 2: Sample Distribution by province.

Blood spotted onto Whatman 3MM filter paper was collected for all microscopically analyzed samples (94). For the remaining samples (237) the used RDTs devices were stored for subsequent nucleic acid extraction.

Samples were collected between 2011 and 2016 with informed consent and approved by the DR institutions: Autonomous University of Santo Domingo, Research Council of the Faculty of Sciences, the National Health Research Department of the Ministry of Health and the IMPA Bioethics Committee. All procedures contributing to this work comply with the ethical standards of the relevant national and

institutional committees on human experimentation and with the Helsinki Declaration of 1964, amended in 2008.

3.1.2. Nucleic acid extraction:

The possibility of recovering DNA from malaria rapid diagnostic tests (RDTs) is validated [87], suggesting that RDTs are a reliable source for DNA preservation. Here, we have extracted DNA from the collected RDTs (237) and from blood spotted filter paper (94).

DNA extraction from filter papers was performed using "NYZ Blood gDNA Isolation Kit" (NYZtech). To extract as much DNA as possible from the RDTs, the same kit was used with initial steps of the protocol modified. The immunochromatographic strips inside the RDTs, were cut into 1-2mm long pieces and soaked in 450 µl of PBS at 4°C for 72hrs. Then, 450µl a of buffer NBL solution with proteinase K (50 µl) was added and incubated at 70°C until the solution changed from a reddish to a dark brown color. Next steps follow the manufactured protocol.

3.1.3. Human G6PD genotyping

Detection of the genetic variant *G6PD A-* (G202A), associated with antimalarial response [88], [89] was done by real-time polymerase chain reaction using TaqMan® SNP Genotyping Assay (SNP ID: rs1050828; Applied Biosystem) on all 331 samples . The reactions were run into a Biorad CFX96 Touch™ Real-Time PCR Detection System. Genotyping assay was performed in a final reaction of 10 µl, containing 5 µl of 2× TaqMan Universal Master Mix, 0.5 µl of 40×TaqMan SNP genotyping assay mix (Applied Biosystems) and 3 µl of genomic DNA in 1,5 µl of distilled water. The amplification conditions were 10 mins at 95°C, followed by 40 cycles of 15 seconds at 95°C for denaturation and 1 min at 60°C for annealing and extension. Allelic discrimination was performed on the post-PCR product and analyzed directly using the Bio-Rad CFX instrument software. Because *G6PD* is linked to X-chromosome, allele frequencies were calculated by dividing the number of samples with allele *G6PD A-* (hemizygote male + homozygote female + heterozygote female) by the total number of genotyped samples.

3.2 Study 2: Detect low-density malaria infections in Dominican Republic

3.2.1 Plasmodium detection

Rapid diagnostic tests and microscopy are less sensitive than molecular diagnosis to detect low parasitemias. In addition, the dip sticks used in Dominican Republic detect only one species (*P.*

falciparum). To determine if *Plasmodium* species where present in the samples presented in 3.1.1., detection and species identification assays were performed based on the protocols established by Weiping , which has a detection sensitivity of 1 parasite/ µl for *P. falciparum* and 2 parasite/ µl for *P. vivax* [30]. Detection was done through amplification of the *Plasmodium cytochrome b* gene by real-time polymerase chain reaction using the KAPA SYBR® FAST qPCR Master Mix (2X) kit (KAPA Biosystems). The reactions were run into a Biorad CFX96 Touch™ Real-Time PCR Detection System in one replicate. The PCR reactions and primer sequences are detailed in tables 3 and 4.

3.2.2 Species identification

Species identification was performed on the post-PCR product of all *cytb* positive samples from 3.2.1 by RFLP with *AluI* restriction enzyme, in a final reaction of 20µL, which contained 7µl of real time PCR product, 1µl of *AluI* restriction enzyme, Thermo Scientific, 2µL of 10 X buffer Tango and 10µL of distilled water [figure 3].

RFLP fragment lengths (bp)

	F	V	M	O
AluI	254 176	270 128 32	398 32	302 128

Figure 3*: Expected band sizes for the different species when digested with AluI restriction enzyme* (F: *P. falciparum*; V: *P. vivax*; M: *P. malariae*; O: *P. ovale) (Modified from* [30]*)*

3.2.3 Detection of *P. falciparum* histidine rich protein 2 (*pfhrp2*) gene deletions

To determine if false negativeness of the RDTs could be due to parasite deletion of the *pfhrp2* gene, a SYBR green real-time polymerase chain reaction was performed on all *cytb* qPCR positive RDT samples from 3.2.1

The primers used were originally designed for end point PCR and published by Ousmane A. Koita [90]. Because of the variability in size of *pfhrp2* [91], all amplifications with melting points different to the negative control's, were considered positive for *pfhrp2*. The reactions were run using the KAPA SYBR® FAST qPCR Master Mix (2X) kit (KAPA Biosystems), on a BioRad CFX96 Touch™ Real-Time PCR Detection System. The PCR reactions and primer sequences are detailed in tables 3 and 4.

Table 3: Primers used in study 2. Detection of low-density malaria infections (*cytB*) and amplification of *pfhrp2*

Primers	Primer sequence (5' -> 3')
***CytB*_Fw (p1)**	tggtagcacaaatcctttaggg
***CytB*_Rv (p2)**	tggtaattgacatccaatcc
***HRP2*_Fw (p3)**	attccgcatttaataataacttgtgtagc
***HRP2*_Rv (p4)**	atggcgtaggcaatgtgtgg

Table 4: PCR reactions used in Study 2 Detection of low-density malaria infections (*cytB*) and amplification of *pfhrp2*

Primers	SYBR green Real time PCR program	Melt curve program	Reaction conditions
***CytB*_Fw; CytB_Rv**	95°C – 4'; 40cycles: 95°C –15'', 60°C – 90'', 72°C - 5'	65°C to 95°C With 0.5°C increments every 5"	5µl of KAPA SYBR® FAST qPCR Master Mix (2X) (KAPA Biosystems), 10µM of each primer and 5µL of genomic DNA in in final reaction of 12 µl.
***hrp2*_Fw; *hrp2*_Rv**	95°C – 4'; 40cycles: 95°C –15'', 60°C – 90'', 72°C - 5'	65°C to 95°C With 0.5°C increments every 5"	5µl of KAPA SYBR® FAST qPCR Master Mix (2X) (KAPA Biosystems), 10µM of each primer and 5µL of genomic DNA in in final reaction of 12 µl.

3.3 Study 3: Prevalence of chloroquine molecular markers of resistance in Dominican parasites

3.3.1 *pfmdr1* and *pfcrt* genotyping

Using the PCR-RFLP [92] and Sanger sequencing, detailed below, *pfmdr1* and *pfcrt* genotyping was done on all the samples from study 2 that were found to be positive for *P. falciparum.*

3.3.1.1 Amplification of *pfmdr1* and *pfcrt*

To obtain enough genetic material for the genotyping of the *pfmdr1* 86th and the *pfcrt* 72nd to 76th amino acid position, a nested and semi nested PCR were performed respectively. For these a fragment of both genes was amplified from clinical samples in two separate initial reactions; followed by nested amplifications using the product from the initial reactions as template. The PCR reactions and primer sequences are detailed in tables 5 and 6.

Table 5: Primers used in study 3. Amplification of *pfmdr1*(nested PCR) and *pfcrt* (semi-nested PCR)

Primers	Primer sequence (5' -> 3')
***pfmdr1*_1st_Fw(P5)**	aagaggttgaaaaagagttgaac
***pfmdr1*_1st_Rv(p6)**	atttcgtaccaattcctgaact
***pfmdr1*_N_Fw(p7)**	agagtaccgctgaattatttag
***pfmdr1*_N_Rv (p8)**	cctgaactcacttgttctaaat
***pfcrt*_Fw (P9)**	tttcccttgtcgaccttaac
***pfcrt*_1st_Rv (p10)**	tgactgaacaggcatctaac
***pfcrt*_N_Rv (p11)**	tggtaggtggaatagattctc

***Table 6**: PCR reactions used in Study 3.* Amplification of *pfmdr1*(nested PCR) and *pfcrt* (semi-nested PCR)

reaction	Primers	SYBR green Real time PCR program	Reaction conditions
1st reaction	*pfmdr1*_1st_Fw; *pfmdr1*_1st_Rv	95°C – 3'; 40cycles: 95°C –30'', 56°C – 30'', 72°C – 60"; 72°C - 10'	1.25U DreamTaq DNA polymerase, (Thermo Scientific), 2.5 µl of 10X DreamTaq buffer, 0.4 µM of each primer, 0.2mM of each dNTP, and 1µl of template DNA in in final reaction of 25 µl.
Nested	*pfmdr1*_N_Fw; *pfmdr1*_N_Rv	95°C – 3'; 40cycles: 95°C –30'', 56°C – 30'', 72°C – 60"; 72°C - 10'	2.5U DreamTaq DNA polymerase, (Thermo Scientific), 5 µl of 10X DreamTaq buffer, 0.8 µM of each primer, 0.4mM of each dNTP, and 0.5µl of template DNA in in final reaction of 25 µl
1st reaction	*pfcrt* _Fw; *pfcrt* _1st_Rv	95°C – 3'; 40cycles: 95°C –30'', 56°C – 30'', 72°C – 60"; 72°C - 10'	1.25U DreamTaq DNA polymerase, (Thermo Scientific), 2.5 µl of 10X DreamTaq buffer, 0.4 µM of each primer, 0.2mM of each dNTP, and 1µl of template DNA in in final reaction of 25 µl
Semi-nested	*pfcrt* _Fw; *pfcrt* _N_Rv	95°C – 3'; 40cycles: 95°C –30'', 47°C – 30'', 72°C – 60"; 72°C - 10'	1.25U DreamTaq DNA polymerase, (Thermo Scientific), 2.5 µl of 10X DreamTaq buffer, 0.4 µM of each primer, 0.2mM of each dNTP, and 1µl of template DNA in in final reaction of 25 µl

3.3.1.2 Restriction fragment length polymorphism (RFLP) for *pfmdr1* N86Y/N86F SNP and *pfcrt* K76T SNP

The resulting amplicons from the nested and semi-nested PCRs were digested using the ApoI restriction enzyme in a final reaction of 20µl, which contained 7µl of nested PCR product, 1µl of XapI (ApoI) restriction enzyme (Thermo Scientific), 2µl of 10 X buffer Tango and 10µl of distilled water. The reaction tubes were incubated for 3 hours at 37°C.

ApoI digests the wild type allele of *pfmdr1* (86N) and leaves the mutant alleles (N86Y and N86F) with decreased sensitivity to chloroquine, undigested. The same thing occurs with the *pfcrt* K76T SNP. The restriction enzyme digests the wild type allele (76K) and leaves the mutant CQ resistant allele (K76T) undigested [figure 4].

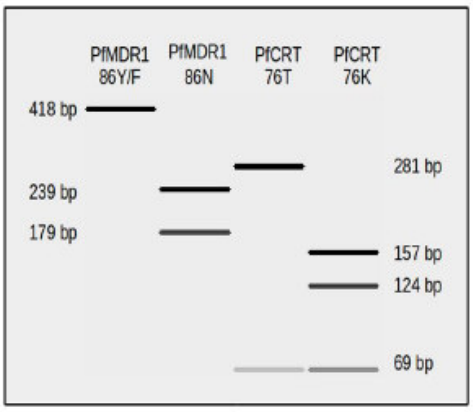

Figure 4 RFLP to genotype *pfcrt* and *pfmdr1* Schematic expected band size of *pfmdr1* and *pfcrt* PCR-RFLP digested with ApoI for the presence of different alleles.

3.3.1.3 *pfmdr1* and *pfcrt* sequencing

After *ApoI* digestion, the *pfmdr1* amplicons that had undigested or incomplete digestion were Sanger sequenced, using the services of Stabvida©. This was done to distinguish between the 86Y and 86F alleles, and to see if mixed infections were present in the samples (86N plus 86Y; 86N plus 86F; 86N plus 86F and/or 86Y; 86Y plus 86F). All the *pfcrt* amplicons were sequenced to determine the amino acids sequence present in positions 72 to 76 of the PfCRT transporter.

3.4 Study 4: Impact of resistance factors

3.4.1 Understanding the interplay of PfCRT and PfMDR1: the role of different PfMDR1 haplotypes in the presence of verapamil.

3.4.1.1. *Plasmodium falciparum* strains used for this study

P. falciparum NF10 strain [table 7], carries a single copy of *pfmdr1*, encoding the N86Y/Y184F mutant haplotype (YF) and encompassing a mutant PfCRT haplotype (CVIET at codons 72–76). From this parent NF10 strain, 4 recombinant parasites lines were previously generated, differing in the PfMDR1 haplotypes (YF, YY, NF, NY) and all carrying the PfCRT CVIET haplotype and herein used for this work [55].

Table 7: Characteristics of the *Plasmodium falciparum* NF10 recombinant lines used in this study.

***P. falciparum* strains**	***pfmdr1* haplotype**		***pfcrt* haplotype**				
	86	**184**	**72**	**73**	**74**	**75**	**76**
NF10_mdr1_NF	N	F	C	V	I	E	T
NF10_mdr1_NY	N	Y	C	V	I	E	T
NF10_mdr1_YY	Y	Y	C	V	I	E	T
NF10_mdr1_YF	Y	F	C	V	I	E	T

3.4.1.1.1. *Plasmodium falciparum* culture

Asexual blood-stage parasites were propagated in human erythrocytes under a controlled atmosphere of 5% O2/5% CO2/90% N2. Cultures flasks were kept at 4% hematocrit in 5 mL of malaria culture medium (MCM) [RPMI 1640 (Gibco) with 2 mM L-glutamine, 200 µM hypoxanthine, 0.25 µg/mL gentamycin, 25 mM HEPES, 0.2% NaHCO3, and 0.25% Albumax II (Life Technologies)]. Medium changes were performed daily – unless otherwise stated – and the cultures healthiness and parasitemia were regularly monitored by microscopy.

3.4.1.1.2 Microscopic analysis and parasitemia assessment

A drop of RBC from the culture flasks is smeared onto a microscopic slide, fixed with 100% methanol and colored with 10% Giemsa stain for 15 minutes. Parasitemia assessment was done under the immersion lens of an optical microscope (amplification 1000X: ocular: 10X; objective 100X), by dividing the number of infected RBC by the total of erythrocytes counted.

3.4.1.4. *Plasmodium falciparum* drug susceptibility assay: SYBR green based fluoroassay

Plasmodium falciparum drug susceptibility assays were performed in flat-bottom 96 well plates in 100 µl of malaria culture medium (MCM), 1% hematocrit and 0.2% starting parasitemia. The plates were pre-dosed with descending concentrations (two fold dilution each consecutive well) of chloroquine, piperaquine, lumefantrine, mefloquine and dihydroartemisinin, with and without verapamil (final concentration 0.8 µM) with initial concentrations of 1000 nM, 250 nM, 25 nM, 75 nM, and 40 nM, respectively. Two wells were left without drug as positive growth control. Verapamil is a calcium channel blocker that inhibits the activity of the PfCRT transporter.[68]

Parasitemia was assessed using a SYBR green based fluoroassay. Each drug susceptibility assay was repeated 3 times, on different dates for each strain.

After 72 hours of culture, plates were frozen at -20°C overnight. In a new 96 flat bottom well plate. 25 µl of lysis buffer [EDTA (10mM), saponin (0.016% w/v, Triton X-100 (1.6% v/v) in sterile water] supplemented with SYBR green 1X, was mixed with 50 µl of culture from each well and incubated at room temperature for three hours in the dark. Then the plates were read on a fluorescence plate reader (Varioskan Flash, Thermo Scientific) with excitation and emission wavelength bands centered at 485 nm and 530 nm, respectively. *In vitro* IC50 values were calculated using nonlinear regression analysis.

3.4.2. Understand the interplay of PfCRT and PfMDR1: PfCRT 72-76 amino acid allele exchange strategy

3.4.2.1. Modifying the NF10 strains: changing the PfCRT haplotype CVIET to CVMNK

To study the interplay of the food vacuole transporters, PfCRT and PfMDR1, a plasmid (pSLI_pfcrt_76K) [figure 5a], needed to be constructed. It was used to replace the CVIET *pfcrt* haplotype into CVMNK on the four isogenic *Plasmodium falciparum* NF10 strains described above [table 7].

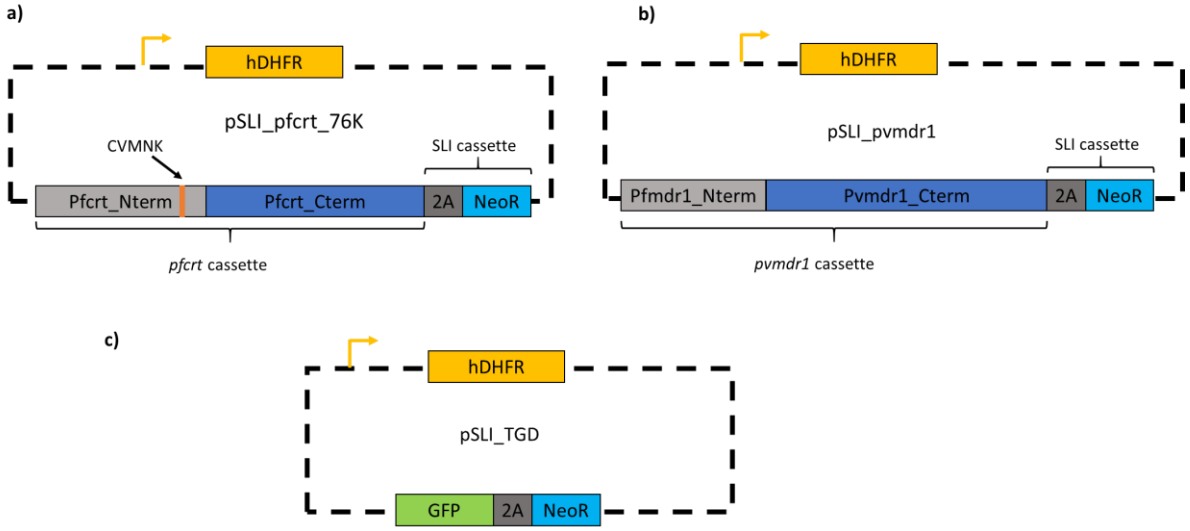

Figure 5: Selection linked integration plasmids. Arrow: promoter; 2A: T2A skip peptide; NeoR: neomycin resistance gene (Integration selection marker); hDHFR human dihydrofolate reductase (plasmid selection marker); **a)** Psli_pfcrt_76K pfcrt_Nterm: Homologous region, from *P. falciparum* 3D7gDNA (764 bp), carrying the desired genetic alteration (CVMNK); pfcrt_Cterm: Non- Homologous region from *P. falciparum* Dd2 cDNA; 2A: T2A skip peptide; NeoR: neomycin resistance gene (Integration selection marker); hDHFR human dihydrofolate reductase (plasmid selection marker); **b)** Psli_pvmdr1: pfmdr1_Nterm: Homologous region from *P. falciparum* 3d7 gDNA (260 bp) ;pvmdr1_Cterm: Non- Homologous region from *P. vivax* clinical isolate gDNA (4152bp); **c)** pSLI_TGD (Addgene plasmid # 85791) plasmid developed by Jakob Birnbaum *et al.* [74].

3.4.2.1.1. *In silico* cloning strategy for constructing the plasmid to modify the NF10 strains

The DNA sequences of *pfcrt* and *pfmdr1* from the *P. falciparum* 3D7 as well as the *pfcrt* from *P. falciparum* Dd2 was obtained from PlasmoDB database (www.plasmodb.org). The plasmid sequence of Psli_TGD, developed by Jakob Birnbaum *et.al.* [74] was obtained from the Addgene database (Addgene plasmid # 85791). The sequence for the Pjet1.2 plasmid (Thermo Scientific) was obtained from the NCBI database (GenBank: EF694056.1). The cloning strategies were designed *in silico* with the software Ape version 2.0.49.

3.4.2.1.2. Building the plasmid to modify the NF10 strains

The first step was to build the *pfcrt* cassette that carries the CVMNK haplotype. A 764bp DNA fragment from *P. falciparum* 3D7 strain genomic was PCR amplified (homologous region) (reaction 1, table 9), as well as an 921bp fragment from *P. falciparum* Dd2 strain complementary DNA (non-homologous region) (reaction 2, table 9). Primers sequences are listed on table 8. The extraction of *P. falciparum* Dd2 RNA and cDNA synthesis are detailed in 3.4.2.1.2.1 and 3.4.2.1.2.2.

Each fragment was cloned separately into the pjet1.2 cloning vector using the "CloneJET" PCR cloning kit, Thermo Scientific™. The pJET 1.2 plasmid contains a lethal gene which is disrupted after ligation, as well as the β-lactamase gene conferring resistance to ampicillin. After transformation, only the bacteria that were transformed with a recombinant plasmid can grow in LB agar containing ampicillin.

For the Homologous region both insert and vector were digested with *HindIII* and *NotI* restriction enzymes. The non-homologous region was cloned into the pjet1.2 cloning vector using the blunt end protocol provided by the manufacturer. The plasmids were then transformed into competent *Escherichia coli*. (see 3.4.2.1.2.3). Nevertheless, colonies were screened by restriction fragment length polymorphism (RFLP) and/or colony PCR, to make sure that both of our fragments integrated into the plasmids in the correct orientation.

After confirmation, both plasmids were digested with *HindIII* and *PvuII* restriction enzymes. The digested products were run on a 1% agarose gel electrophoresis the 934 bp band from the digested pJET plasmid containing the non-homologous region of the cassette, and the 3163 bp band digested pJET plasmid containing the homologous region of the cassette, were extracted from the gel using the GRS PCR & Gel Band Purification Kit, GRISP research Solutions, and ligated with T4 DNA ligase, Thermo Scientific. The resulting plasmid containing thus the *pfcrt* cassette (See figure 6). The plasmid was transformed into competent *E. coli*.

Table 8: Primers used in the construction of pSLI_crt76K and Psli_pvmdr1 plasmids;

Primers	Primer sequence (5' -> 3')
***pfcrt*_Nterm_Fwd (p12)**	atactcgcggccgc**atg**aaattcgcaagtaaaaaaaataa
****pfcrt*_Nterm_Rev (p13)**	caaaaaattgtaaattaaagcttcggtgtcgttcctaaaa
***pfcrt*_Cterm_Fwd (p14)**	ttttaggaacgacaccgaagctttaatttacaatttttg
***pfcrt*_Cterm_Rev (p15)**	gacttgtgtaataattgaatcgacgttggttaattctcct
***pfmdr1*_Nterm_ Fwd (p16)**	atactcgcggccgcatgggtaaagagcagaaagagaaaaa
***pfmdr1*_Nterm_ Rev (p17)**	agttcatattcttcataatcttaagtattacaccaaacac
***pvmdr1*_Cterm_ Fwd (p18)**	gtgtttggtgtaatacttaagattatgaagaatatgaact
***pvmdr1*_Cterm_ rev (p19)**	ggacggcgtatataagaagtacgtcaagctggctaaggtc

**Underline: Not restriction site; Bold: start codon; ** underline:* HindIII *restriction site.*

Table 9: PCR reactions used in the construction of pSli_crt 76K and pSli_pvmdr1 plasmids

Reaction	Primers	PCR program	Reaction conditions
1	*pfcrt*_Nterm_Fwd; *Pfcrt*_Nterm_Rev	95°C – 3'; 35 cycles: 95°C - 15'', 63°C - 15'', 72°C – 30"; 72°C - 10'	0.5 U of KAPA HiFi HotStart Ready mix©KAPA biosystems, 0.3 µ M of each primer, 1 µ l of template DNA in final reaction of 25 µl.
2	*pfcrt*_Cterm_Fwd; *pfcrt*_Cterm_Rev	98°C – 30"; 35 cycles: 98°C - 10'', 55°C - 20'', 72°C – 30"; 72°C - 10'	2 U of Phusion Hot Start II High-Fidelity DNA Polymerase Thermo scientific, 0.5 µ M of each primer; 200 µ M of each dNTP; 1 µ L of template cDNA; 10 µ l of 5X Phusion HF Buffer; 1.5 mM of MgCl in final reaction of 50 µl.
3	*pfcrt*_Nterm_Fwd; *pfcrt*_Cterm_Rev	98°C – 30"; 35 cycles: 98°C - 10'', 55°C - 20'', 72°C – 2'; 72°C - 10'	2 U of Phusion Hot Start II High-Fidelity DNA Polymerase Thermo scientific, 0.5 µ M of each primer; 200 µ M of each dNTP; 1 µ l of template pJet_*pfcrt*_76K; 10 µ L of 5X Phusion HF Buffer; 1.5 mM of MgCl in final reaction of 50 µl.
4	*pfmdr1*_Nterm_ Fwd; *pfmdr1*_Nterm_ Rev	98°C – 30"; 35 cycles: 98°C - 10'', 55°C - 20'', 72°C – 15"; 72°C - 10'	2 U of Phusion Hot Start II High-Fidelity DNA Polymerase Thermo scientific, 0.5 µ M of each primer; 200 µ M of each dNTP; 0.5 µ l of template P. *falciparum* 3D7 DNA, 4 µl of 5X Phusion HF Buffer, in final reaction of 20 µl.
5	*pvmdr*1_Cterm_ Fwd; *pvmdr1*_Cterm_ rev	98°C – 30"; 35 cycles: 98°C - 10'', 55°C - 20'', 72°C – 2'; 72°C - 10'	2 U of Phusion Hot Start II High-Fidelity DNA Polymerase Thermo scientific; 0.5 µ M of each primer; 200 µ M of each dNTP; 3 µ L of template *P. vivax* clinical Isolate DNA, 10 µl of 5X Phusion HF buffer, in in final reaction of 50 µl.
6	*pfmdr*1_Nterm_ Fwd; *pvmdr*1_Cterm_ rev	98°C – 30"; 35 cycles: 98°C - 10'', 55°C - 20'', 72°C – 5'; 72°C - 10'	2 U of Phusion Hot Start II High-Fidelity DNA Polymerase Thermo scientific; 0.5 µ M of each primer; 200 µ M of each dNTP; 1 µ L of template pJet_*pvmdr1;* 10 µl of 5X Phusion HF buffer, in in final reaction of 50 µl.

The PCR product was then restricted using *NotI* restriction enzyme and purified using the GRS PCR & Gel Band Purification Kit, GRISP research Solutions. The pSLI_TGD backbone was restricted with *NotI* and *HincII* Restriction enzymes. Generating a blunt end on the *HincII* side and a sticky end on the *NotI* side. The digestion product of the pSLI_TGD was run in a 1% agarose gel and the desired band (5994bp) was extracted from gel using the GRS PCR & Gel Band Purification Kit, GRISP research Solutions. Both fragments were ligated with T4 DNA ligase and transformed into competent *E. coli*.

Colonies where screened by *Hind III* RFLP as well as a *HincII* RFLP after enrichment (LB broth supplemented with ampicillin) and plasmid extraction (NZYMiniprep, plasmid extraction kit, NZYtech). The selected plasmid [figures 5a and 6] was confirmed by Sanger sequence.

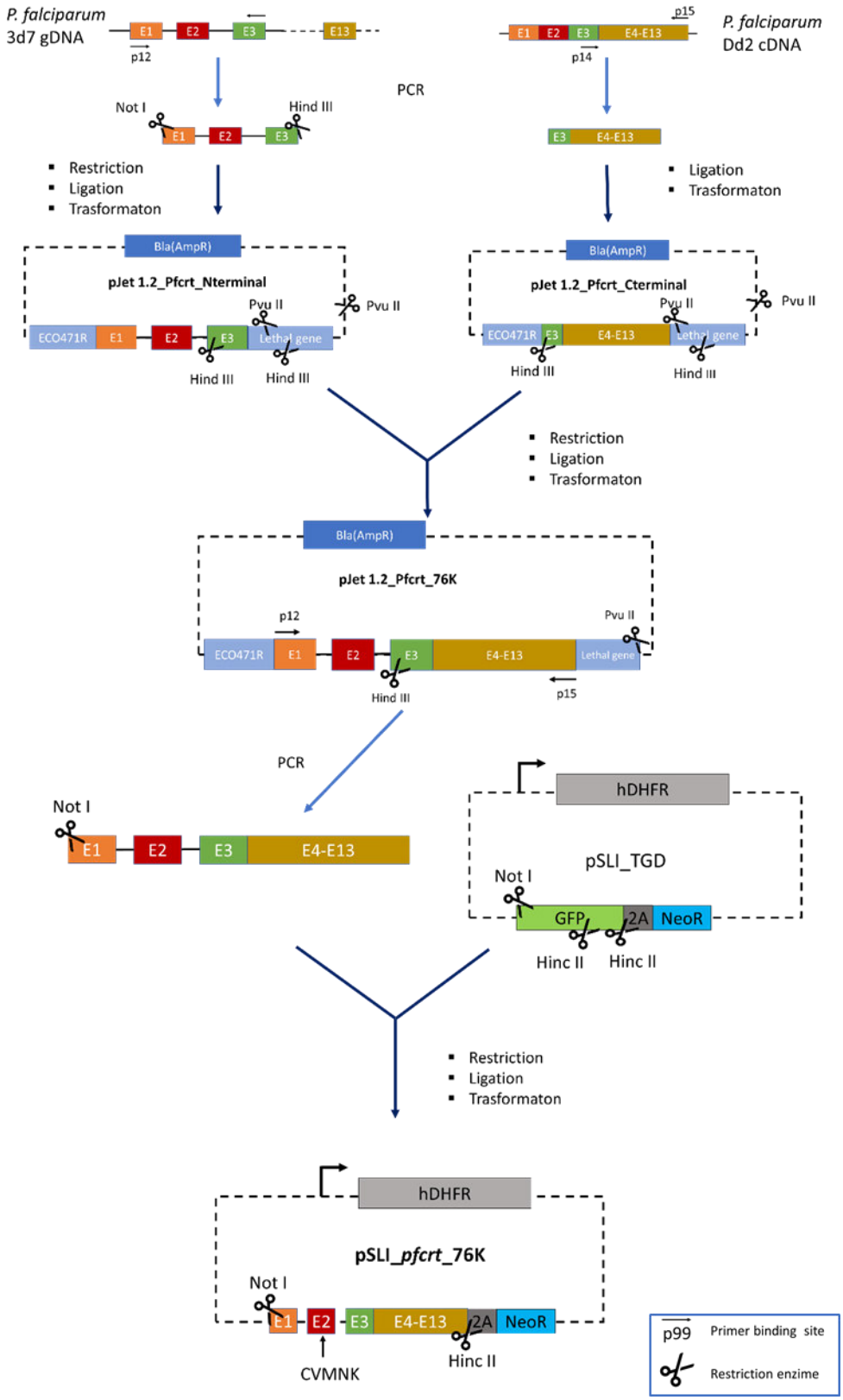

Figure 6 : Building the pSLI_pfcrt_76K plasmid Pjet 1.2_Pfcrt_Nterminal: intermediate plasmid with homologous region insert; Pjet1.2_pfcrt_Cterminal: intermediate plasmid with non-homologous region insert; pJet_pfcrt_76K: intermediate plasmid with pfcrt_76K cassette ; pSLI_TGD *: backbone* for final plasmid*;pSli_pfcrt_76K,* plasmid to modify the *4 P. falciparum* NF10 strains *pfcrt* haplotype from CVIET to CVMNK

3.4.2.1.2.1. RNA extraction

P. falciparum cultures (parasitemia > 4%) were centrifuged at 4600 rpm for 5 minutes with no brake to collect the infected red blood cells (RBC). The RBCs were then washed with PBS (10 X volume) and centrifuged again at 4600 rpm for 5 minutes with no brake. The supernatant was discarded, and the RBC lysed by freezing at -80°C for twenty minutes thawing them in a water bath at 65 °C for 2 to 3 minutes. NZYol regent (NZYtech) was added (10 x volume) and the cells were resuspended vigorously shaking the tube until no clumps remain. Chloroform was added to the mix (1/5 of the NZYol volume), followed by vigorous shaking of the tube. The sample was then incubated for 5 minutes on ice with intermittent shaking, then centrifuged for 30 minutes at 4600 rpm at 4 °C.

The top clear aqueous phase was transferred to a clean tube, mixed with equal volume of isopropanol 100% and incubated overnight at -20°C. Next the tube was centrifuged at 4600 rpm at 4 °C for 1 to 2 hours. The pellet was then washed with the same volume of 70% ethanol as the volume of NZYol during the initial extraction and dislodged from the walls by inverting the tube several times. After 10 minutes incubation on ice, the sample was centrifuged at 4600 rpm at 4 °C for 15 minutes. The supernatant was removed completely with a pipette, and the pellet was resuspended in 15–30 ul of nuclease free water.

The RNA samples were stored at -80°C. the quality of RNA was checked by running an aliquot of the sample on a 1% denaturing gel at 7 V/cm for 1–2 h. There should be three distinct bands observed at 3.8 kb (28S rRNA), 2.0 kb (18S rRNA), and 160 bp (5.8S rRNA).

3.4.2.1.2.2. Complementary DNA synthesis.

Complementary DNA synthesis using NZY first strand cDNA synthesis kit, NZYtech, following the manufactures instruction.

3.4.2.1.2.3. Plasmid transformation into *Escherichia coli*

Transformation of *E. coli* DH5 α competent cells was performed by adding 5 µ L of each ligation reaction to 50 µ L of bacterial solution. This was followed by an incubation of 15 min on ice and then heat shocking the mix at 42°C for 35 seconds. The mix was immediately placed on ice. Afterward, in order to increase the number of E. coli cells carrying the plasmid before selection, the solution was incubated at 37°C (200rpm shaking) for 1 to 2 hours in 300 µl of super optimal broth (SOB) medium [0.5% (w/v) yeast extract, 2% 30 (w/v) tryptone, 10 mM NaCl, 2.5 mM KCl, 20 mM MgSO4]. The solution was then

inoculated into Luria-Bertani (LB) agar plates [10 g/L bacto-tryptone, 5 g/L yeast extract ,10 g/L NaCl and 15g/L agar] supplemented with ampicillin overnight. After transformation, only the bacteria that were transformed with a recombinant plasmid can grow in LB agar containing ampicillin.

3.4.2.1.2.4. Plasmid DNA isolation

To obtain plasmid DNA quantities of up to 10 μg, at concentrations ranging from 100 ng/μl to 500 ng/μl, plasmid DNA was isolated using a modified version of the alkaline lysis method [93] or the NZYminiprep plasmid extraction kit, NZYtech.

The modified alkaline lysis method was used mainly for colony screening purposes. Colonies were expanded into 3 mL of LB liquid medium, supplemented with the ampicillin, at 37°C, with shaking, overnight. The culture was then centrifuged at 8000 rpm for 2 minutes and the cell pellet was resuspended with 250 μl of P1 solution. Then 250 μl of P2 solution was added, mixed by inversion and incubated for 5 minutes at RT. Next 300 μl of P3 solution was added, mixed by inversion and centrifuged at 14000 rpm for 5 minutes. Five hundred microliters of isopropanol 100% was added to the supernatant, mixed and centrifuged at 12000 rpm for 5 minutes. The pellet was then washed with 200 μl of ethanol 70% and centrifuged at 12000 rpm for 5 minutes. The supernatant was completely removed by evaporation and the plasmid DNA pellet was resuspended in 30 μl of nuclease free water Plasmid DNA extracted using NZYminiprep kit was performed following manufacturer recommendations. It was used when subsequent manipulations of the plasmids were required.

For transfection into *P. falciparum*, plasmid DNA quantities of up to 50μg, at concentrations of 1 μg/μl, per transfection were needed. In these cases, the NZYmaxiprep endotoxin free plasmid extraction kit,NZYtech, which has a yield of up to 500μg of DNA, was used following manufacturer recommendations.

3.4.2.1.2.5. DNA restrictions

DNA restrictions were used to prepare DNA inserts and vectors for cloning. Different enzymes were used to create either sticky or blunt ends depending on the case. Restrictions were also used analytically to confirm proper integration of the desired fragments into the vectors.

All restriction enzymes used in this study where Anza restriction enzymes Thermo Scientific™. Digestions were performed according to the manufacturer's instructions.

3.4.2.3 Getting recombinant *P. falciparum* NF10 strains

3.4.2.3.1 Parasite transfection

An attempt to transfect the four *P. falciparum* NF10 recombinant lines (table 7) with the pSLI_pfcrt_76K plasmid was made. The transfection process was performed by electroporation of the plasmid mixture, which contains 50 μg of each plasmid to transfect in 300 μl of cytomix, into 200 μl of infected erythrocytes (parasitemia 4% to 5%). The infected erythrocytes mixed with plasmids were transferred to Gene Pulser®/MicroPulser™ Electroporation Cuvettes, 0.2 cm gap (BioRad) and the electroporation was performed at 0.31 kV with capacitance of 950 μfD on the Gene Pulser Xcell™ (BioRad) electroporator. After the electroporation, the mixture was placed into a culture flask containing medium and after one hour, washed five times in 1 mL of malaria culture medium (MCM) [RPMI 1640 (Gibco) with 2 mM L-glutamine, 200 μM hypoxanthine, 0.25 μg/mL gentamycin, 25 mM HEPES, 0.2% NaHCO3, and 0.25% Albumax II (Life Technologies)] and transferred to 25 cm^2 culture flasks (4 % hematocrit, in 5 ml of MCM).

3.4.2.3.2 Parasite selection

Asexual blood-stage parasites were propagated in human erythrocytes under a controlled atmosphere of 5% O2/5% CO2/90% N2. Cultures flasks were kept at 4% hematocrit in 5 mL of MCM, Medium changes were performed daily – unless otherwise stated – and the cultures healthiness and parasitemia were regularly monitored by microscopy (see 3.4.1.1.2).

Given that the human dihydrofolate reductase gene (*hdhfr*) is a selectable marker present in the pSli_pfcrt_76K, [figure 5a] confers resistance to WR99210. After 24 hours of culture (see 3.4.1.1.1), MCM was supplemented with WR99210, Jacobs Pharmaceuticals. (final concentration, 0.5nM), to select for parasites expressing the plasmids episomally. The cultures healthiness and parasitemia were regularly monitored by microscopy. During this first stage of selection, parasitemia decreases before it starts to increase again.

To take advantage of the selection linked integration system, which carries a neomycin resistance selection marker, linked to the pfcrt_76K, that can only be expressed once the plasmid has been integrated into the parasite genome after homologous recombination. Once parasitemia has reached 4% again, the second stage of selection started. This time with G418, sulfate, EMD Millipore Corp. (final concentration 400ug/ml). Because The pSli plasmid has no promoter for the *pfcrt* cassette-Sli cassette complex [figure 5a], only parasites that have integrated the plasmid can grow under these conditions. The cultures healthiness and parasitemia were regularly monitored by microscopy.

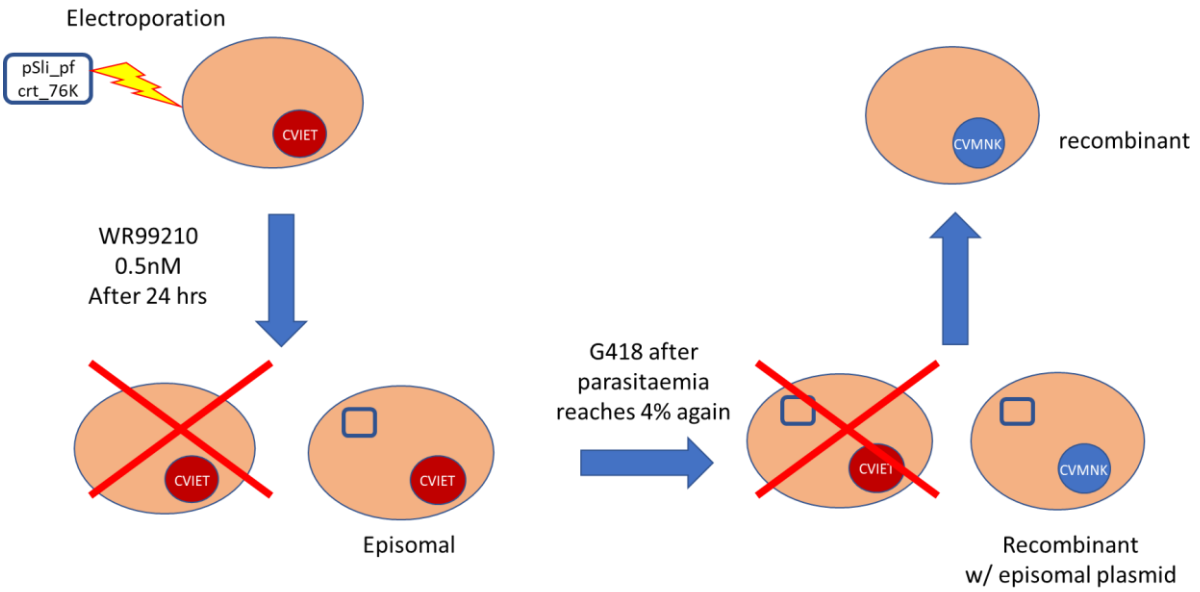

Figure 7: Illustration of the selection process of recombinant *P. falciparum* NF10 parasites in culture.

3.4.2.3.2 Confirm integration of Psli_pfcrt_76K on *Plasmodium falciparum* NF10 recombinant lines

Blood PCRs were done (blood from the culture flasks are used as template), to verify the 5' integration of the psli_pfcrt_76K plasmid into on the transfected parasites. To check if the *pfcrt* gene was edited as intended (if the *pfcrt* CVIET haplotype was replaced by CVMNK haplotype). To genotype the modified NF10 parasites, a second PCR was performed on the product from the previous reaction, followed by an *Apo*I RFLP. The expected bands after RFLP are showed in figure 4a. The primers used and the reactions are described on tables 10 and 11 and figure 8.

Table 10: Primers to confirm the integration pSLI_crt76K into the *P. falciparum* genome.

Primers	Primer sequence (5' -> 3')
***pfcrt*_5'UTR_Fw (p20)**	ccgttaataataaatacacgcag
****pfcrt*_int_Rv (p21)**	caagaccacaattctctacgactg
*****Pfcrt*_WT_RV (p22)**	agcttcttacCCATGCTCCGTC
***pfcrt*_Fw (p9)**	tggtaggtggaatagattctc
***pfcrt*_N_Rv (p11)**	tgactgaacaggcatctaac

*the *pfcrt*_int_Rv primer was designed as an exon-exon primer spanning over intron 7 to ensure that only our constructed pfcrt would be amplified *in reaction1.*** *The Pfcrt*_WT_RV *only binds to intact pfcrt.*, they were all designed using the Ape software version 2.0.49.

Table 11: PCR reactions used to confirm the integration of Psli_pfcrt_76K on *Plasmodium falciparum* NF10 parasites

Reaction	Primers	PCR program	Reaction conditions
1	*Pfcrt*_5'UTR_Fw; *Pfcrt*_int_Rv *Pfcrt*_WT_RV	95°C – 5' ; 35 cycles : 94°C - 30'', 57.1°C - 20'', 72°C – 15" ; 72°C - 10'	10µL of Supreme NZYTaq II 2× Green Master Mix, NZYtech, 0.5 µM of each primer; 5µL blood from the cultures as template, in a final reaction of 20 µl.
2	CRT_Fw; CRT_N_Rv	95°C – 3' ; 35 cycles : 94°C - 30'', 63.4°C - 20'', 72°C – 1' ; 72°C - 10'	10µL of NZYTaq II 2× Green Master Mix, NZYtech, 0.5 µM of each primer; 5µL from the product of reaction 1 as template, in final reaction of 20 µl.

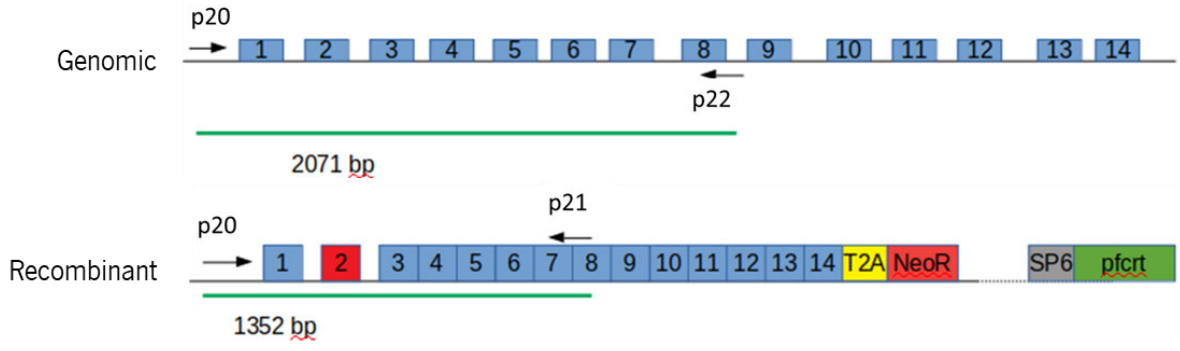

Figure 8 Illustration of confirmation PCR primers binding sites. Primer p20 binds to the 5' UTR of the *pfcrt* gene; primer p21 and p22 bind specifically to the *pfcrt* cassette and the NF10 *pfcrt* respectively. The green lines represent the expected amplicons and their sizes. Numbered squares represent exons, exon 2 is where the 76th aa position is located.

3.4.3 The generation of a hybrid Pf/Pv MDR1 as a concept to study *P. vivax* resistance genes using *P. falciparum* as a model.

3.4.3.1. Modifying *Plasmodium falciparum* 3D7: replacing *pfmdr1* by *pvmdr1*

3.4.3.1.1. Building the plasmid to modify the NF10 strains

To build the pSLI_*pvmdr1* construct [figure5b], the same technological approach used in section 3.4.2 was used, with some differences:

- The *pvmdr1* sequences from *P. vivax* Sal1 were obtained from PlasmoDB (www.plasmodb.org).

- DNA extraction from *Plasmodium vivax* clinical isolate (blood spotted filter paper) according to the protocol described in section 3.1.2.

- The 260 bp homologous region of the *pvmdr1* cassette was amplified from the *P. falciparum* 3D7 *pfmdr1* genomic DNA and the 4152 bp non-homologous region from a *P. vivax* clinical isolate genomic DNA [tables 8 and 9].

- To make sure that both of our fragments integrated into the plasmids in the correct orientation. The screening of *E. coli* colonies carrying the intermediate plasmids were also done by RFLP and/or colony PCR, the main differences being that both plasmids were digested with *AflII* and *XbaI* restriction enzymes.

-The bands that were extracted from the agarose gel after electrophoresis were, an 4151bp band from the pJet 1.2 plasmid containing the *pvmdr1* non homologous region and a 3226 bp band from the one containing the homologous region from *pfmdr1* before ligation and transformation to obtain the intermediate plasmid containing the *pvmdr1* cassette. (pJet 1.2_*pvmdr1*)

- The *pvmdr1* cassette was PCR amplified from the pJet 1.2_*pvmdr1* plasmid [reaction 6, table 9], and ligated into the pSLI_TGD backbone in the same manner as described in section 3.4.2.1.2.

- Colonies were screened by colony PCR.

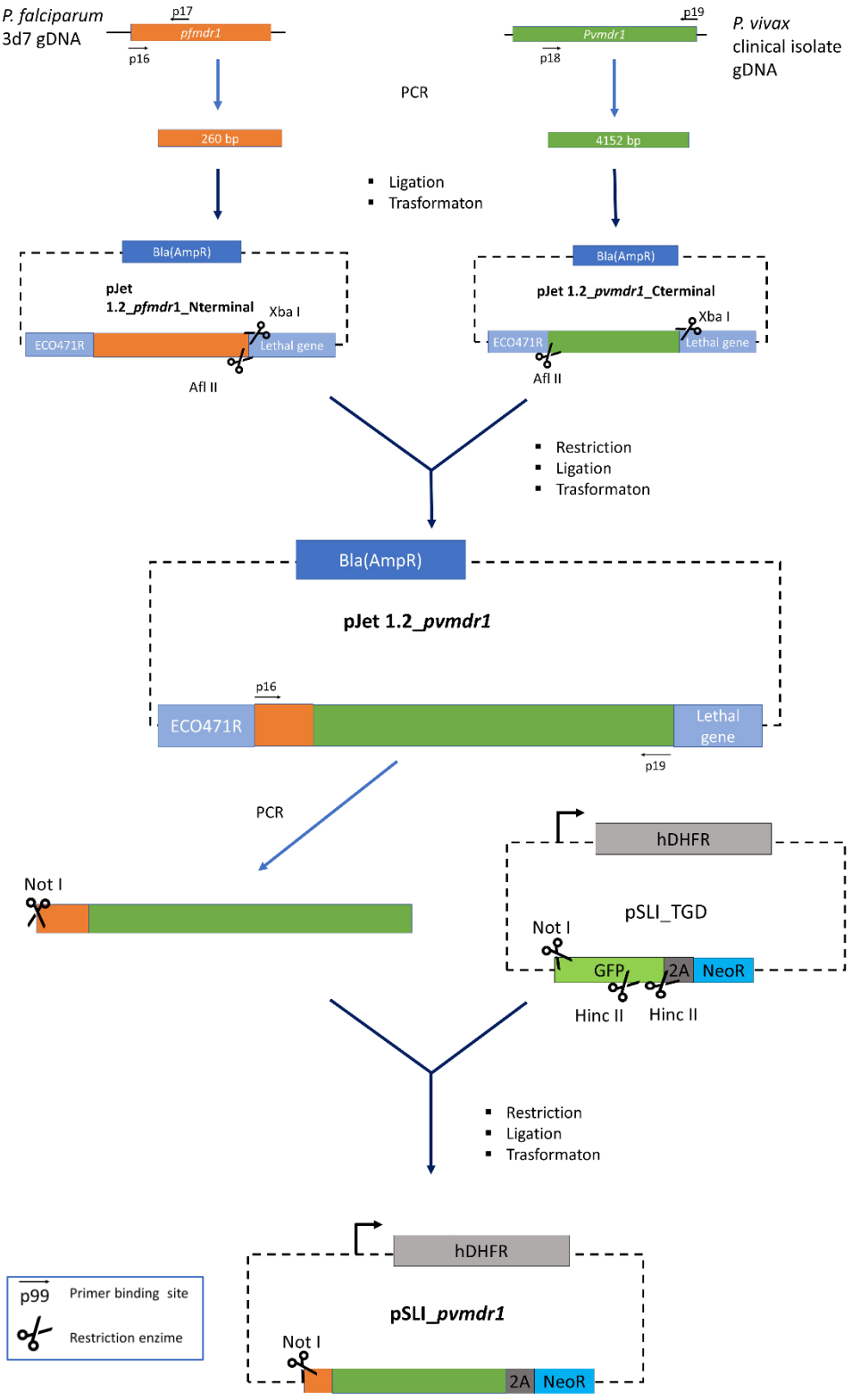

Figure 9 Building the pSLI_*pvmdr1* plasmid.; Plasmids: Pjet 1.2_*pfmdr1*_Nterminal: intermediate plasmid with homologous region insert; Pjet1.2_*pvmdr1*_C terminal: intermediate plasmid with non-homologous region insert; pJet_*pvmdr1*.: intermediate plasmid with pvmdr1cassette ; pSLI_TGD : backbone for final plasmid; pSLI_pvmdr1, plasmid to modify replace *pfmdr1* by *pvmdr1* in *P. falciparum* 3D7

3.4.3.2 Getting hybrid *P. falciparum* 3D7

3.4.3.2.1 Parasite transfection

This step was done in same manner as described on 3.4.2.3.1, the only differences being the transfected parasite strain was *P. falciparum* 3D7 because it carries only one copy of *pfmdr1* and the plasmid (pSLI_*pvmdr1*).

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3.4.2.3.2 Parasite selection

Given that the same technological approach as in with the NF10 strains was used the selection process here was the same.

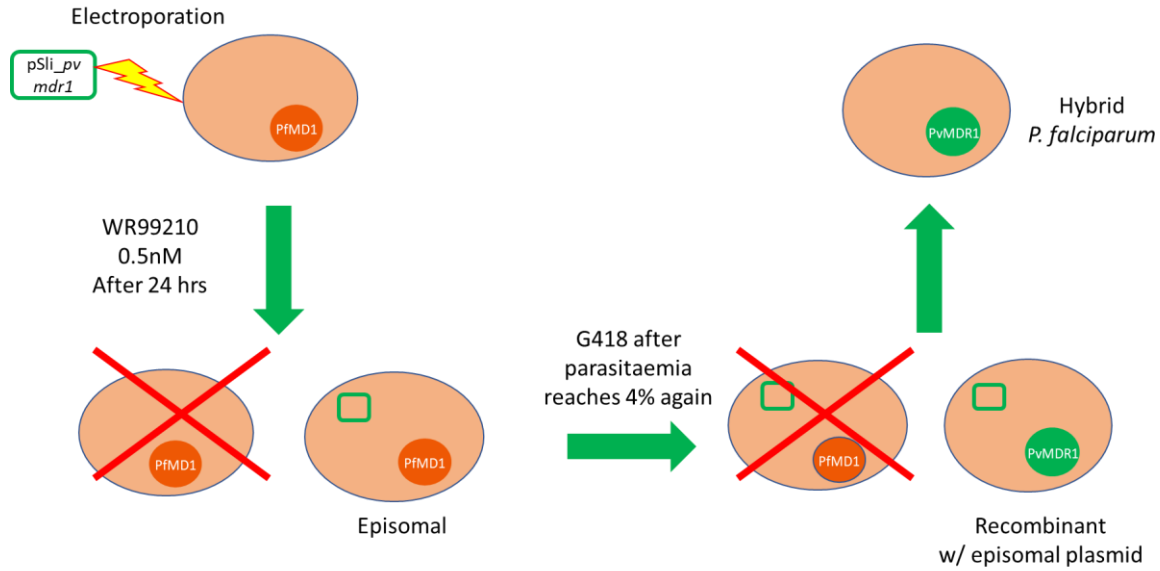

Figure 10: Illustration of the selection process of hybrid *P. falciparum* 3D7 parasites in culture.

3.4.3.3.3 Confirm integration of Psli_pfcrt_76K on *Plasmodium falciparum* NF10 parasites

To verify the 5' integration of the psli_pvmdr1 plasmid into on the transfected parasites. Two blood PCRs were performed one to check for integration and the second as a control detect the *pfmdr1* gene. The primers used and the reactions are described on tables 12 and 13 and figure 11.

Table 12: Primers to confirm the integration pSLI_pvmdr1 into the *P. falciparum* 3D7 genome.*the *Pf3D7*_5'UTR_Fw primer was designed bind into the *Plasmodium falciparum* 3D7 5' UTR; ** *The Pfmdr1*_WT_RV only binds the intact *pfmdr1* and *Pvmdr1*_int_Rv only binds to an *pvmdr1* site present on our construct. They were all designed using the Ape software version 2.0.49.

Primers	Primer sequence (5' -> 3')
***Pf3D7*_5'UTR_Fw (p23)**	acattttatttgattttgtgttga
***Pvmdr1*_int_Rv (p24)**	tttaatttcttcccgtcctgg
***Pfmdr1*_WT_Rv (p25)**	cttattacatatgacaccacaaac

Table 13: PCR reactions used to confirm integration the integration of pSLI_*pvmdr1* into the *P. falciparum* 3D7 genome

Reaction	Primers	PCR program	Reaction conditions
1	*Pf3D7_5'UTR_Fw*; *Pfmdr1_WT_Rv*	95°C – 3' ; 35 cycles : 94°C - 30'', 48°C - 30'', 72°C – 1' ; 72°C - 10'	10µL of NZYTaq II 2× Green Master Mix, NZYtech, 0.5 µM of each primer; 5µL from the product of reaction 1 as template, in final reaction of 20 µl.
2	*Pf3D7_5'UTR_Fw*; *Pvmdr1_int_Rv*	95°C – 5' ; 35 cycles : 94°C - 30'', 55°C - 20'', 72°C – 15" ; 72°C - 10'	10µL of Supreme NZYTaq II 2× Green Master Mix, NZYtech, 0.5 µM of each primer; 5µL blood from the cultures as template, in a final reaction of 20 µl.

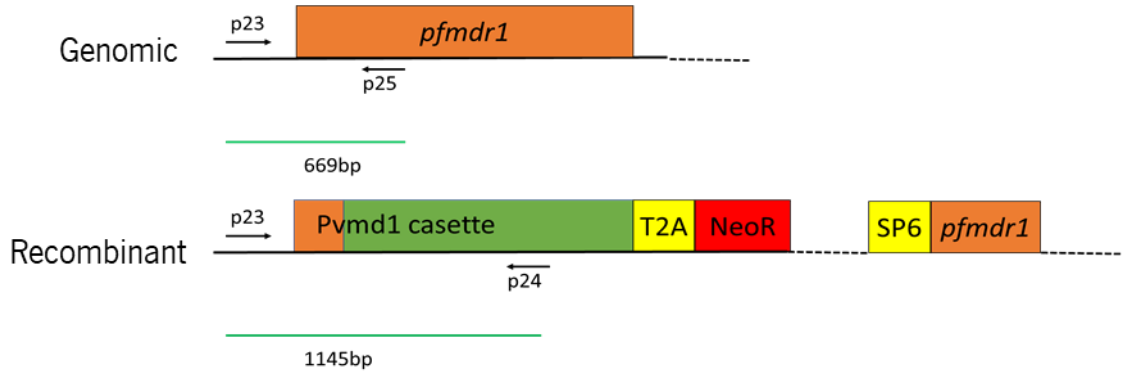

Figure 11: Illustration of confirmation PCR primers binding sites. Primer p23 binds to the 5' UTR of the *pfmdr1* gene; primer p24 and p25 bind specifically to the *pvmdr1* cassette and the the 3D7 *pfmdr1* respectively. The green lines represent the expected amplicons and their sizes.

***Pfmdr1 copy number variation assay* gene on hybrid *P. falciparum* 3D7.**

The primers used were originally designed probe base multipex qPCR and published by R. Price *et al.*[94]. For the purpose of this study the assay was adapted to a double- simplex SYBR green based assay. The *β-tubulin* gene used was as reference and *P. falciparum* 3D7 strain as the calibrator. The reactions were done in triplicate, using the KAPA SYBR® FAST qPCR Master Mix (2X) kit (KAPA Biosystems), on a BioRad

CFX96 Touch™ Real-Time PCR Detection System. The PCR reactions and primer sequences are detailed in tables 14 and 15. The gene copy numbers were determined using the $2^{-\Delta\Delta Ct}$ method.

Table 14 : Primers used in *pfmdr1* copy number variation assay

Primers	**Primer sequence (5' -> 3')**
CNV_*pfmdr1*_Fw	tcgtgtgttccatgtgactgt
CNV_*pfmdr1*_RV	tttaataaccctgatcgaaatggaacctttg
*β-tubulin*_Fw	tgatgtgcgcaagtgatcc
*β-tubulin*_Rv	tcctttgtggacattcttcctc

Table 15: PCR reactions used in *pfmdr1* copy number variation assay

Primers	**SYBR green Real time PCR program**	**Melt curve program**	**Reaction conditions**
CNV_*pfmdr1*_Fw**;** CNV_*pfmdr1*_Rv	95°C – 3'; 50cycles: 95°C –15'', 58°C – 1', 72°C - 5'	65°C to 95°C With 0.5°C increments every 5"	5µL of KAPA SYBR® FAST qPCR Master Mix (2X) (KAPA Biosystems), 10µM of each primer and 5µL of genomic DNA in in final reaction of 12 µl.
*β-tubulin*_Fw; *β-tubulin*_Rv	95°C – 4'; 40cycles: 95°C –15'', 60°C – 90'', 72°C - 5'	65°C to 95°C With 0.5°C increments every 5"	5µL of KAPA SYBR® FAST qPCR Master Mix (2X) (KAPA Biosystems), 10µM of each primer and 5µL of genomic DNA in in final reaction of 12 µl.

4. Results and Discussion

4.1 Study 1: Prevalence of G6PD genetic variants in Dominican Republic

The *G6PD A-* variant was successfully genotyped in all samples (331/331). Frequencies and geographical distribution of the samples are shown in table 16. The *G6PD A-* allele was present in 21.11% of the population (70 samples) with 19 (5.7%) Dominicans being male hemizygous or female homozygous and 51 (15.4%) Dominican females being heterozygous.

Table 16: Geographical distribution of *g6pd* genotypes

Province	# sample	Hemizygous male / Homozygous female	Heterozygous female	Normal male / female
Barahona	318	17	46	256
Santo Domingo	8	1	4	8
La Altagracia	2	1	0	1
Baoruco	1	0	0	1
La Vega	1	0	0	1
San José de Ocóa	1	0	1	0
TOTAL	331	19	51	272

G6PD deficiency interferes with the enzyme's role in providing a reductive shield to the RBC through a supply of NADPH. As a consequence, it can lead to hemolytic anemia upon exposure to oxidative medication, such as primaquine, with severity dependent on the dose of the drug and the degree of deficiency [91].

Primaquine is the essential co-drug with chloroquine in treating all cases of malaria. Primaquine also enhances the efficacy of chloroquine. It is highly effective against the gametocytes of all plasmodia and thereby prevents spread of the disease to the mosquito from the patient. It has insignificant activity against the asexual blood forms of the parasite and therefore, to treat *P. falciparum,* it is used in conjunction with a blood schizonticide such as chloroquine or an ACT. But it is not practical neither feasible to test each and every patient for G6PD deficiency before administering primaquine. To overcome this, the World Health Organization (WHO) recommends the use of single low-dose primaquine (SLD-PQ) (0.25mg/Kg) shown to be safe even in patients with G6PD deficiency [95].

In Dominican Republic the primaquine dose used is 0.75mg/Kg, three time higher than the recommended without checking for G6PD deficiency. Our results shows that one in five Dominicans harbours the G6PD A- variant alerting for the potential risk of hemolytic anemia in this subject if

primaquine is taken. Nevertheless up to today, there is no reports of hemolytic effect upon primaquine uptake in the country.

Chen *et al*. assessed the safety of 0.40 and 0.50 mg/kg SLD-PQ usage in glucose-6-phosphate dehydrogenase (G6PD) deficient adult males in Mali [50], providing evidence that extending the upper bound of the therapeutic dose range of SLD-PQ is possible. Taken together, the experience in DR, where the recommended first-line treatment for uncomplicated *P. falciparum* malaria includes a 0.75 mg/kg SLD-PQ together with chloroquine , with no alarming secondary effects reported or known to occur due to this regimen[38], our results, revealing a high proportion of G6PD A- variant, also support the extension of the upper bound of the therapeutic dose range of SLD-PQ with successful clinical results. [51]

4.2 Study 2: Genotyping negative samples confirmed by RDTs or microscopy

4.2.1 Plasmodium detection and species identification

In 66 out of the 331 samples, *Plasmodium spp.* was detected by real time PCR [figure 12; table 17], all of which turned out to be *P. falciparum* after AluI RFLP [figure 13; table17]. Looking at the results by sample type, 20 out of the 94 microscopically analyzed and 46 of the 237 RDTs were positive for malaria [table 17]. These results show that 19.4% of the samples analyzed by RDT and 21.3% of the microscopically analyzed ones were false negatives.

Table 17*: Parasite detection and species identification. RDTs (-): Rapid Diagnostic test negative samples; Smear (-): filter paper from microscopically negative samples; Plasmodium spp.: samples where Plasmodium spp. was detected by CytB qPCR; P. falciparum: species identified after AluI RFLP*

Sample	Total	*Plasmodium spp.*	*P. falciparum*
Smear (-)	94	20	20
RDT (-)	237	46	46
Total	331	66	66

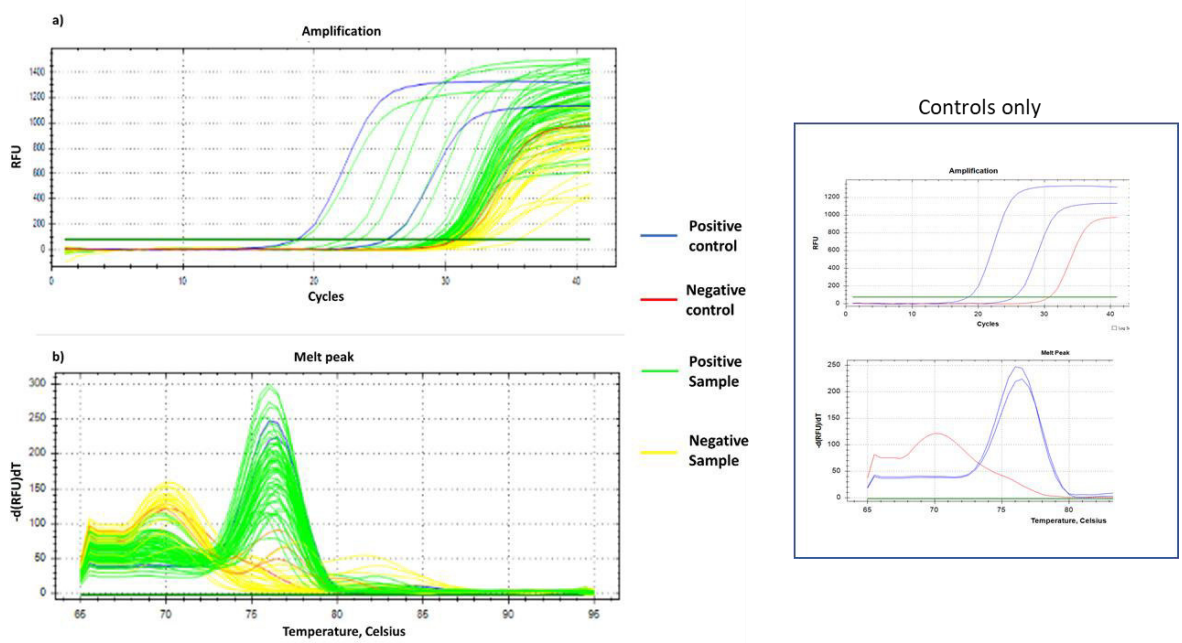

Figure 12: *Plasmodium spp.* detection by SYBR green real time PCR; a) Amplification plot; b) melting curve analysis.

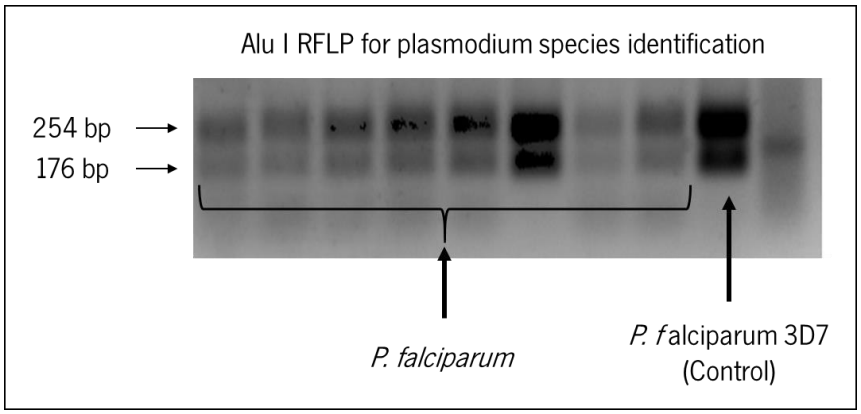

Figure 13: *Plasmodium* species identification RFLP. Agarose gel electrophoresis of all positives Cytochrome B Real time PCR product after digestion with Alu I restriction enzyme. P falciparum 3D7 strain was used as a positive control, all the samples show two bands at 250 bp and 176 bp after digestion indicative of *Plasmodium falciparum*

The RDTs used in DR namely, *First Response® Malaria Antigen P. falciparum (HRP2) Card Test* (Premier Medical Corporation Limited), are based on the principle of immunochromatography using monoclonal antibody specific to Histidine Rich Protein 2 (HRP2) of the *Plasmodium falciparum*. While this type of HRP2 RDTs generally have the highest sensitivity of the RDTs for *P. falciparum* malaria, parasite strains have recently been identified that have deletions in the genes encoding HRP2 [90][96][97]. We have tested if the cause of false-negative RDT results could be due to the absence of this gene in the parasites from Dominican Republic. *Pfhrp2* was detected in 40 out of the 46 false negative RDTs [Figure 14;table

18]. In the remaining 6 we couldn't determine if negative results were due to gene deletion, or due to very low parasite DNA.

Table 18 *pfhrp2* detection SYBR green real time PCR performed on false negative RDTs

	RDTs False negative Samples
***pfhrp2* (+)**	40
***pfhrp2* (-)**	6
Total	46

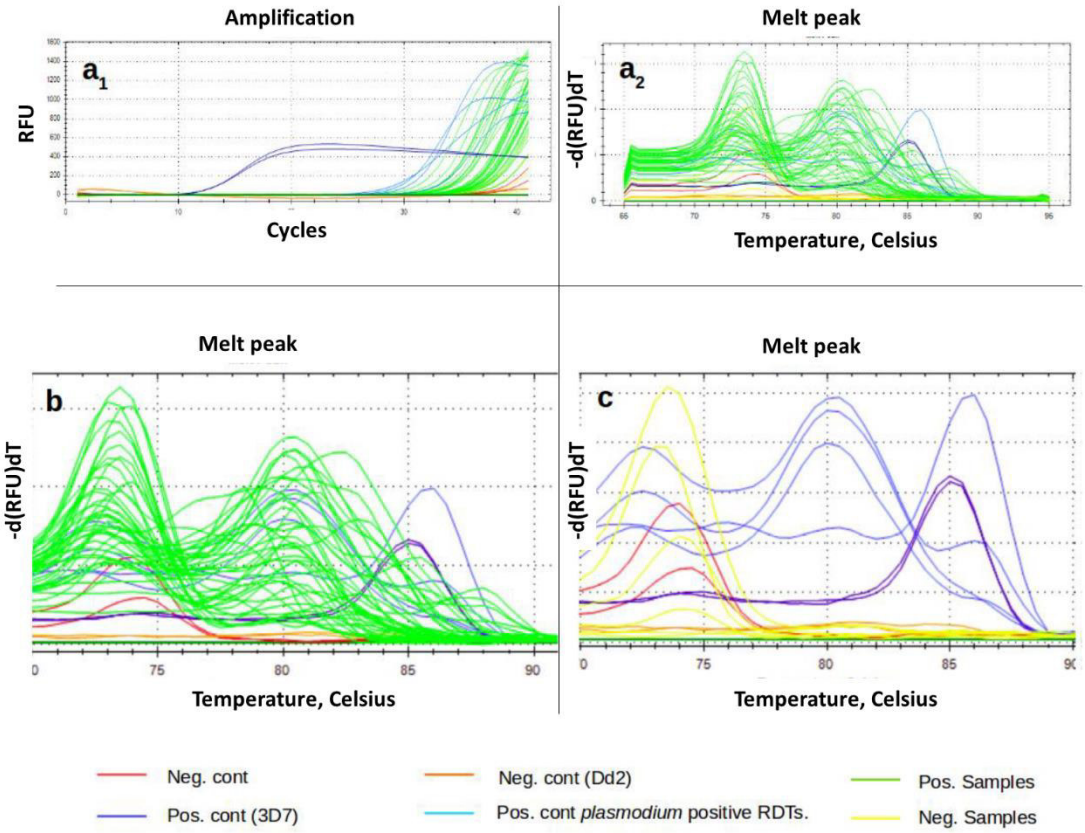

Figure 14 *Pfhrp2* SYBR green real time PCR; **a1)** All samples: amplification plot; **a2)** All samples, melting curve analysis ; b) positive samples, melting curve analysis. All samples with melt peaks above 75°C are considered positive due to the variability in size of the pfhrp2 gene (green); c) negative samples melt curves. Samples with melt peaks between 73.5°C and 74.5°C are considered negative (yellow).

The proportion of negative RDTs where *pfhrp2* was amplified (86.9%) tells that the number of false negative tests cannot be explained by *pfhrp2* deletions. The WHO has been promoting the study of *pfhrp2* deletions. In a recent publication "Protocol for estimating the prevalence of *pfhrp2*/ *pfhrp3* gene deletions

among symptomatic falciparum patients with false-negative RDT results", that deletions in the Peruvian Amazon between 2003 and 2005, showed an increase in prevalence of 40%, they also mention data from Erythria showing an estimated prevalence of hrp2 deletion of 80%. It also notes that prevalence of *pfhrp2* deletions is varies between countries [98].The fact that in our study we couldn't determine the presence these deletions in the Dominican Republic does not mean that they shouldn't be monitored. It is important to note the difference of sensitivity of the different diagnostic methods, microscopy goes up to 50 parasites/μL, RDTs 100 parasites/μL , and the molecular methods used here can detect p. falciparum with a sensitivity of 1 parasites/μL [26] [29][30] . This taken together with the fact that the proportion of false negative RDTs is almost the same as in microscopically analyzed samples indicates that we are in a situation where patients are being symptomatic with very low levels of parasitemia.

By 2030 Under a strategic plan towards malaria elimination, an accurate malaria diagnosis is essential. Our results show that in this regard the Dominican Republic is no different. Standard diagnosis such as microscopy or antigen recognition, might be failing at detecting sub-patent infections. Clearing this parasite reservoir is critical for malaria elimination as these individuals remain infectious to the mosquito promoting the disease transmission. The fact that, using molecular tools we were able to detect *Plasmodium falciparum* in instances where thick smear and dip sticks were unsuccessful tells the need to implement more sensitive tools to strengthen malaria elimination programs. Nevertheless, the subset of samples we have used are not representative of the samples diagnosed in the country. According to the WHO, in 2017 38 547, samples were examined by RDT and 226 988 samples were examined by microscopy, all 398 malaria cases reported that year were confirmed by microscopy [1]. These results need to be confirmed through a much bigger study.

4.3 Study 3: Prevalence of chloroquine molecular markers of resistance in Dominican parasites

4.3.1 *Pfmdr1* and *pfcrt* genotyping

Pfmdr1 was successfully amplified in 42 (63.6%) out of the 66 positive samples. Seven out of these were 86N, 12 were 86Y and 9 had a rare genotype, 86F. Fourteen samples had mixed infections. Three had a mix of the three alleles,1 had a mix of 86N and 86F, 7 had a mix of 86N and 86Y and 3 samples had a mix of alleles 86Y and 86F. (Figure15; Table 19]

Table 19 *pfmdr1* amplifications and genotypes

pfmdr1								
86N	**86Y**	**86F**	**N/F**	**N/Y**	**Y/F**	**N/Y/F**	**Not amplified**	**Total**
7	12	9	1	7	3	3	24	66

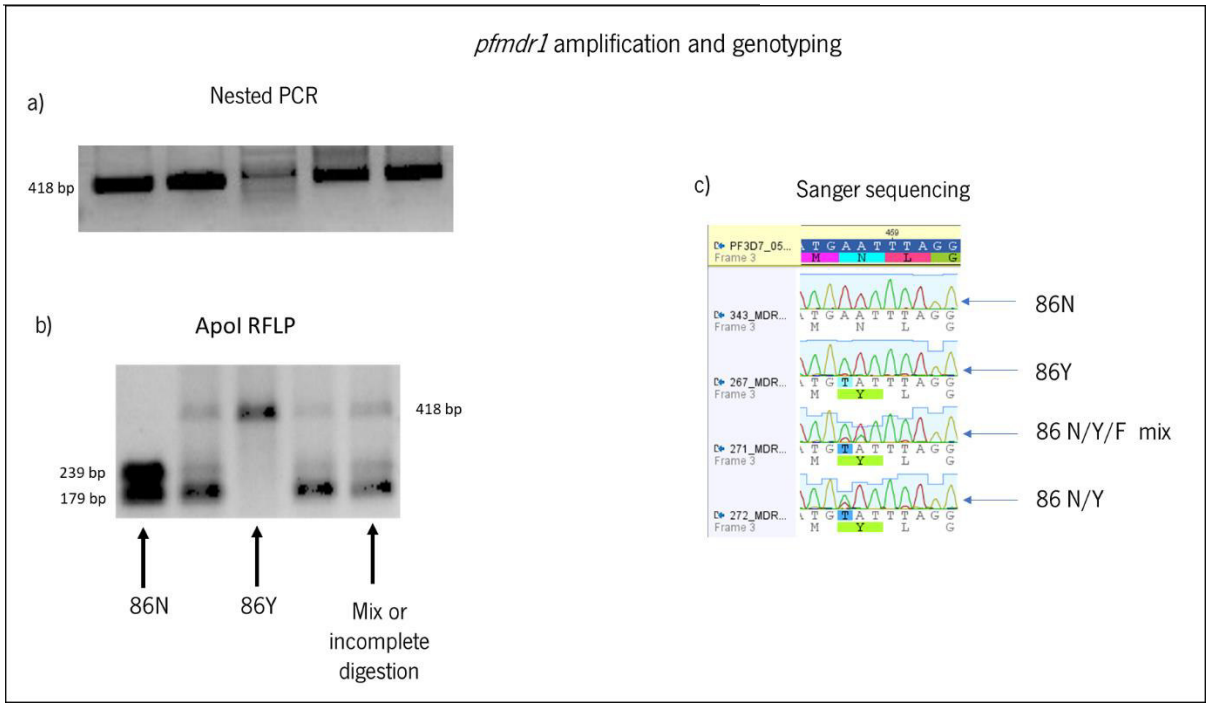

Figure 15 *Pfmdr1* amplification and genotyping; **a)** Agarose gel electrophoresis on *pfmdr1* nested PCR product (418 bp); **b)** Agarose gel electrophoresis of amplified *pfmdr1* PCR product after digestion with ApoI restriction enzyme; **c)** Sanger sequencing on *pfmdr1* PCR product. The sequences where aligned to *P. falciparum* 3D7 *pfmdr1* sequence. The samples shown here are representative of the types of samples found in this study

Similarly, *pfcrt* was amplified in 31 (47.0%) out of the 66 positive samples. Out of those, 29 samples were CVIET, 1 was CVMNK and 1 had a mix infection [Figure 16; Table 20]

Table 20 : *pfcrt* amplifications and genotypes

pfcrt				
CVIET	**CVMNK**	**CVIET/CVMNK**	**Not amplified**	**Total**
29	1	1	35	66

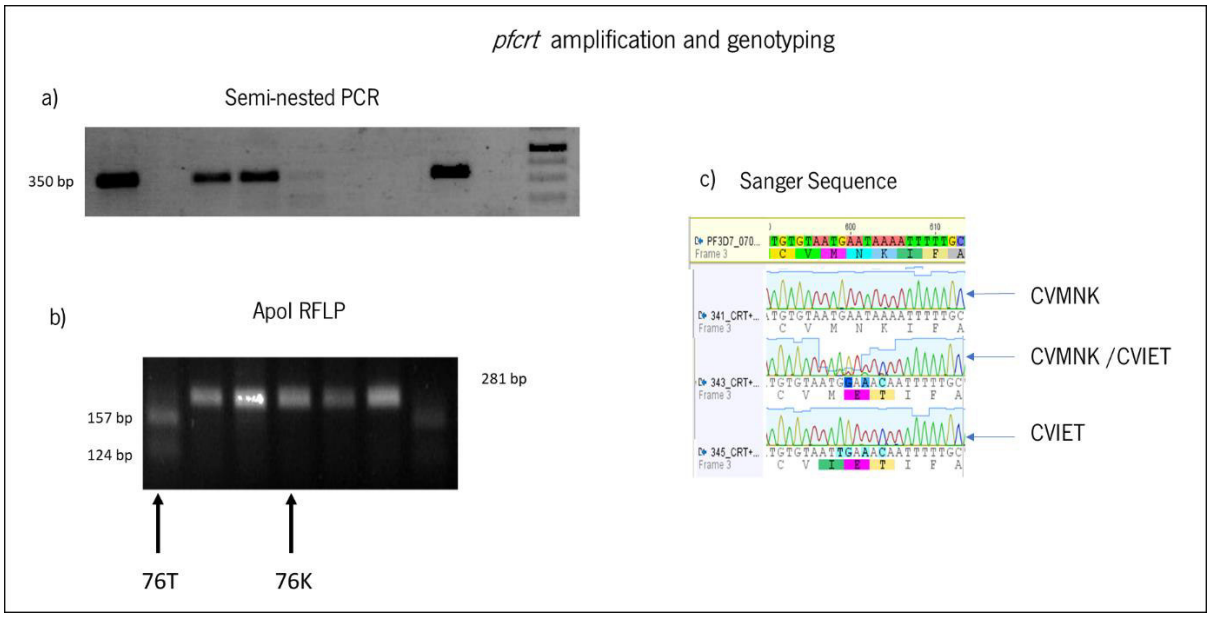

Figure 16 *Pfcrt* amplification and genotyping; **a)** Agarose gel electrophoresis on *pfcrt* semi nested PCR product (350 bp); **b)** Agarose gel electrophoresis of amplified *pfcrt* PCR product after digestion with ApoI restriction enzyme; **c)** Sanger sequencing on *pfcrt* PCR product. The sequences where aligned to *P. falciparum* 3D7 *pfcrt* sequence. The samples shown here are representative of the three types of samples found in this study.

In 26 cases amplification and genotyping of both *pfcrt* 72nd to 76th amino acid positions and *pfmdr1* 86th position was successful, allowing the analysis the haplotypes present in these samples. Among these 26 samples 16 had single strain infections [dark yellow cells, table 21]. The other10 had mixed infections. [light yellow cells, table 21]. Taking mixed infections into account, within the parasite population in our samples, we found five different genotypes, CVIET/86Y, CVIET/86F, CVIET/86N, CVMNK/86N and CVMNK/86F.

Table 21 *pfcrt* and *pfmdr1* amplification and genotyping results

Haplotypes		***pfcrt***				
		CVIET	**CVMNK**	**CVIET/CVMNK mix**	**Not amplified**	**Total**
pfmdr1	**86N**	4	1	1	1	7
	86Y	5	0	0	7	12
	86F	6	0	0	3	9
	N/F mix	1	0	0	0	1
	N/Y mix	5	0	0	2	7
	Y/F mix	2	0	0	1	3
	N/Y/F mix	1	0	0	2	3
	Not amplified	5	0	0	19	24
	Total	29	1	1	35	66

Even though, clinical failure of chloroquine has not been reported in Dominican Republic, molecular markers of resistance to chloroquine were found in the parasite population that was studied. This is the first study on molecular analyses of parasite genetics made in DR to assess antimalarial drug resistance revealing cautious for the current malaria elimination program running in DR.

Looking at the *pfcrt* haplotypes, CVIET, the one that has been more strongly linked to CQ resistance, was present in 96.7% (30 /31) of the instances where *pfcrt* was successfully amplified.

So why is there no clinical failure to CQ the DR?

Given that our sample set is comprised of low parasitemia subjects, which would not be medicated until parasites are detected by the techniques used in the country. One possible explanation might be the difference in parasite fitness between wild type and resistant parasites. Under no selective drug pressure, malaria parasites that carry resistance-mediating polymorphisms could be out-competed by wild type parasites[99].

Another hypothesis is that primaquine enhances the efficacy of chloroquine. And together with chloroquine PQ is used as the first line treatment for uncomplicated malaria in the Dominican Republic [38]. In vitro studies done by P. G. Bray *et. al. in* 2005 with laboratory adapted strains showed that "PQ acts as a synergist of CQ against CQ resistant *P. falciparum,* and appears to be PfCRT-dependent" [100].

The possibility that patients are being given a higher than recommend dose of chloroquine might also be a possibility .Even if we have no evidence that this is happening in DR there is precedent of this occurring in Guinea-Bissau, where patients were given double and even triple the recommended dose of chloroquine with evidence of being efficacious against *P. falciparum* carrying 76T *pfcrt* mutation [101].

These hypotheses need to be explored with further studies to truly understand what is happening in the DR regarding CQ resistance or lack thereof.

4.4. Study 4: Impact of resistance factors

4.4.1 The interplay of PfCRT and PfMDR1, understanding the role of PfMDR1 by inhibiting PfCRT using verapamil.

Our results show that the sensitivity to chloroquine of all four NF10 *P. falciparum* strains is substantially increased in the presence of verapamil. Given that verapamil blocks the PfCRT transporter we can confirm that *pfcrt* CVIET haplotype is the dominant factor in conferring chloroquine resistance to *P. falciparum*, as shown by Veiga *et. al.* study that used this same strains[55].

The drug assays for piperaquine (PPQ) and dihydroartemisinin (DHA) showed no difference in inhibitory concentrations in the presence of verapamil vis a vis the absence of it.

Another interesting finding is that the lumenfantrine (LUM) and mefloquine (MQ) assays, both drugs being of the arylaminoalcohol family, the NF10 86N /184 Y (NY) and NF10 86N /184 F (NF), show a different response in the presence of verapamil. There is a slight increase in the sensitivity mefloquine and no apparent change in the inhibitory concentration of piperaquine. This suggests that even though both drugs are related their mechanism of action might be different. To confirm this more repetitions of the assays must be performed.

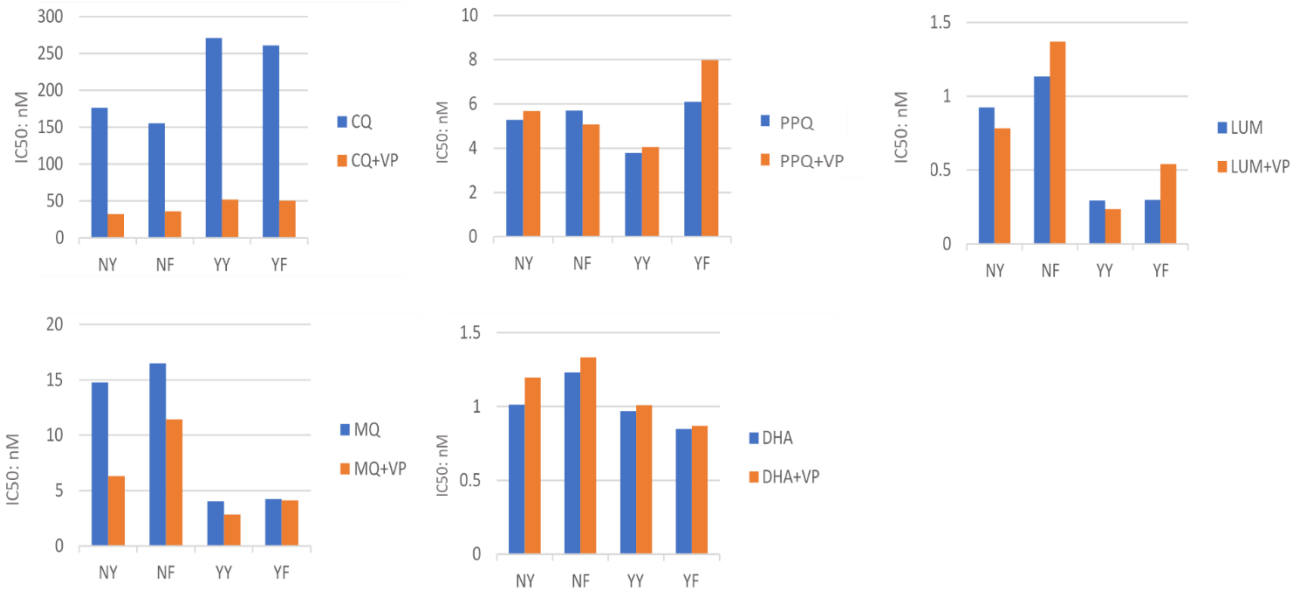

Figure 17: Drug susceptibility assays on *P. falciparum* NF10 recombinant lines in the presence or absence of verapamil (0.8 µM) CQ: Chloroquine; PPQ: piperaquine ; LUM: Lumenfantrine; MQ: mefloquine; DHA: Dihydroartemisinin; VP: Verapamil; In the y axis of the graphs the IC50 s are represented. The assays were performed only once.

4.4.2 Modifying the NF10 strains: changing the *pfcrt* haplotype CVIET to CVMNK

The plasmid to modify the four NF10 *P. falciparum* parasites was successfully constructed. [Figure 18] shows the results of the step by step verifications that lead to the obtention of the final pSli-pfcrt-76K plasmid.

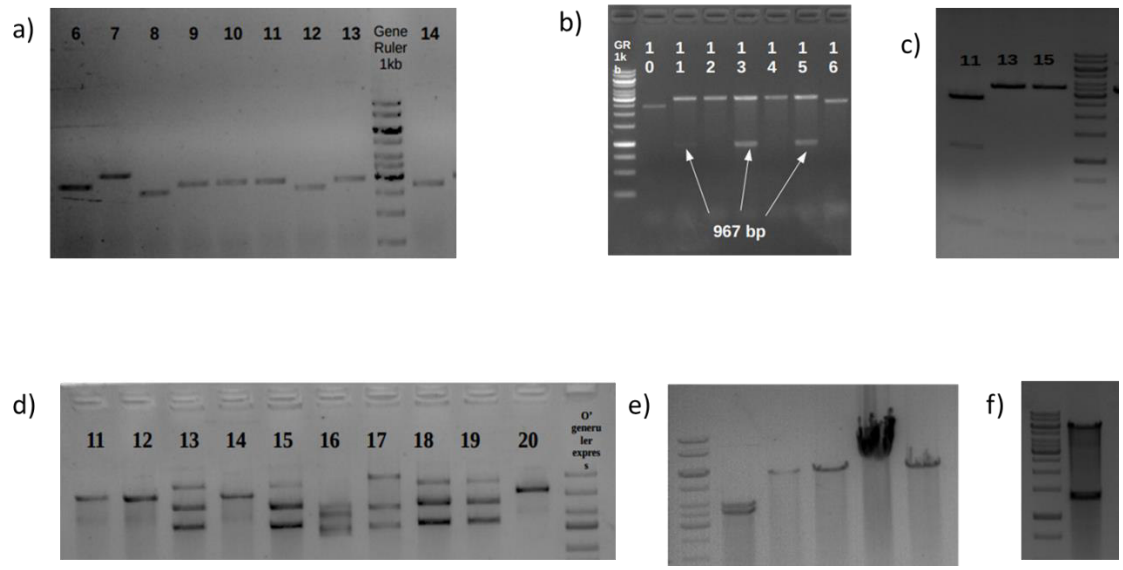

Figure 18: Building the plasmid to modify the NF10 strains **a)** Directional screening of of pJET_pfcrt_Nterminal Hind III rflp: colony 7 correct; **b)** initial screening of pJET _pfcrt_C terminal, BglII RFLP; **c)** directional sreening of pJet_pfcrt_C terminal. HindII RFLP colony 11 correct; **d)** pJET pfcrt screaning XhoI + XbaI RFLP Coloinies 13,15,17,18,19 correct (partial digestion xpected bands (2427bp and 1670 bp); **e)** final Plasmid (pSli-pfcrt-76K) confirmation initial screening hindIII RFLP; **f)** final Plasmid confirmation Hinc II RFLP

After transfection culture and selection of the NF10 parasites (see figure 7), the parasitemias of the cultures were followed. Only the NF10 YY and the NF10 YF were successfully transfected, given that they're the only ones that grew after selection with WR99210 [figure 19a]. Both parasites were able to row under G418 drug pressure [figure 19 b and c], evidence that the pSli-pfcrt-76K plasmid successfully integrated into the genomes of the aforementioned *P. falciparum* strains.

Integration of the plasmid into NF10YY and NF10 YF was confirmed by PCR [figure 20]. After genotyping these parasites using a combination of PCR and RFLP the desired genetic modifications couldn't be observed [figure 21]. Moreover, all batches of parasites show the 76T *pfcrt* genotype. Only one of the batches of NF10 YF shows a possible mix of 76T and 76K [figure 21, YFK3].

To select for the NF10 YF, 76K parasites an extra step of selection by cloning would have to be done, defeating one of the advantages of the selection linked integration approach. Despite the mixed results the repurposing of the SLI technique shows promise as a tool for genetic recombination in *P. falciparum.*

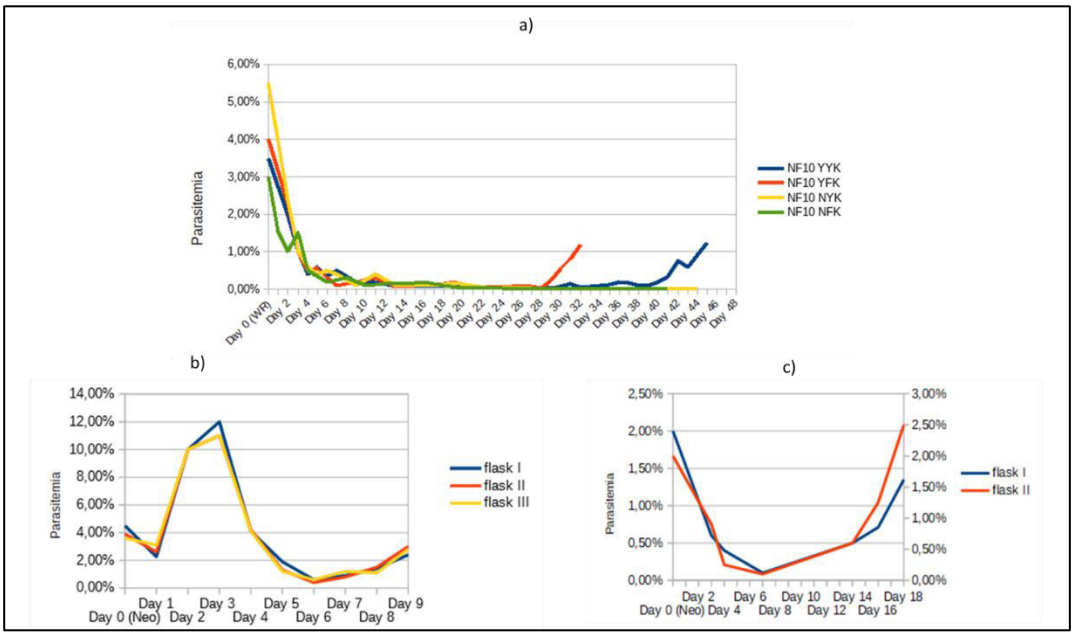

Figure19: parasite selection : **a)** selecion for *Plasmodium falciparum* NF10 parasites carring episomal plasmid (pSLI_pfcrt_76K) with WR 0.5 nM; **b)** NF10_ YFK : selection for integration of plasmid into genome with Neomycin 400nM ; **c)** NF10_ YYK : selection for integration of plasmid into genome with Neomycin 400nM

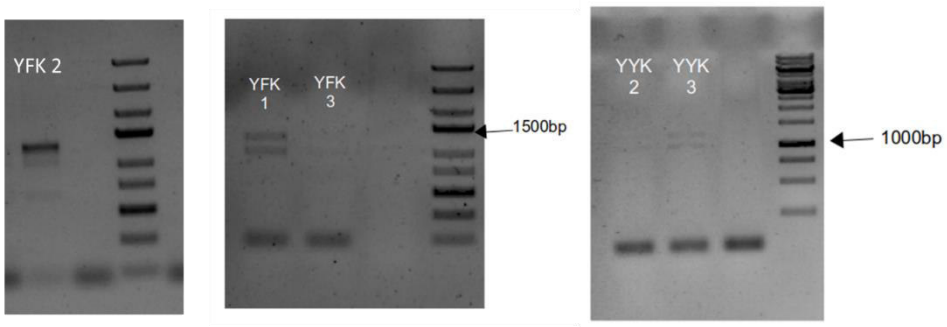

Figure 20: *Integration confirmation:* Integration PCR on NF10 YFK, and NF10 YYK (expected band size 1352bp)

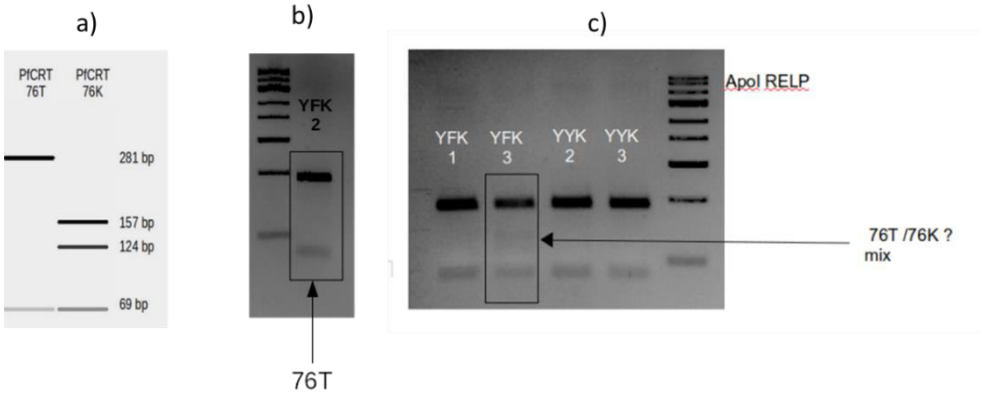

Figure 21: Integration confirmation: Genotypification of NF10 integrants on integration PCR products By ApoI RFLP after nested pcr; a) in silico model; **b)** and **c)** RFLP results for parasites that successfully grew after selection with Neomycin

4.4.3 The generation of a hybrid Pf/Pv MDR1 as a concept to study *P. vivax* resistance genes using *P. falciparum* as a model.

The plasmid to replace *pfmdr1* by *pvmdr1* in *P. falciparum* 3D7 was successfully constructed. Figure 22 shows the results of the step by step verifications that lead to the final pSli-pvmdr1 plasmid.

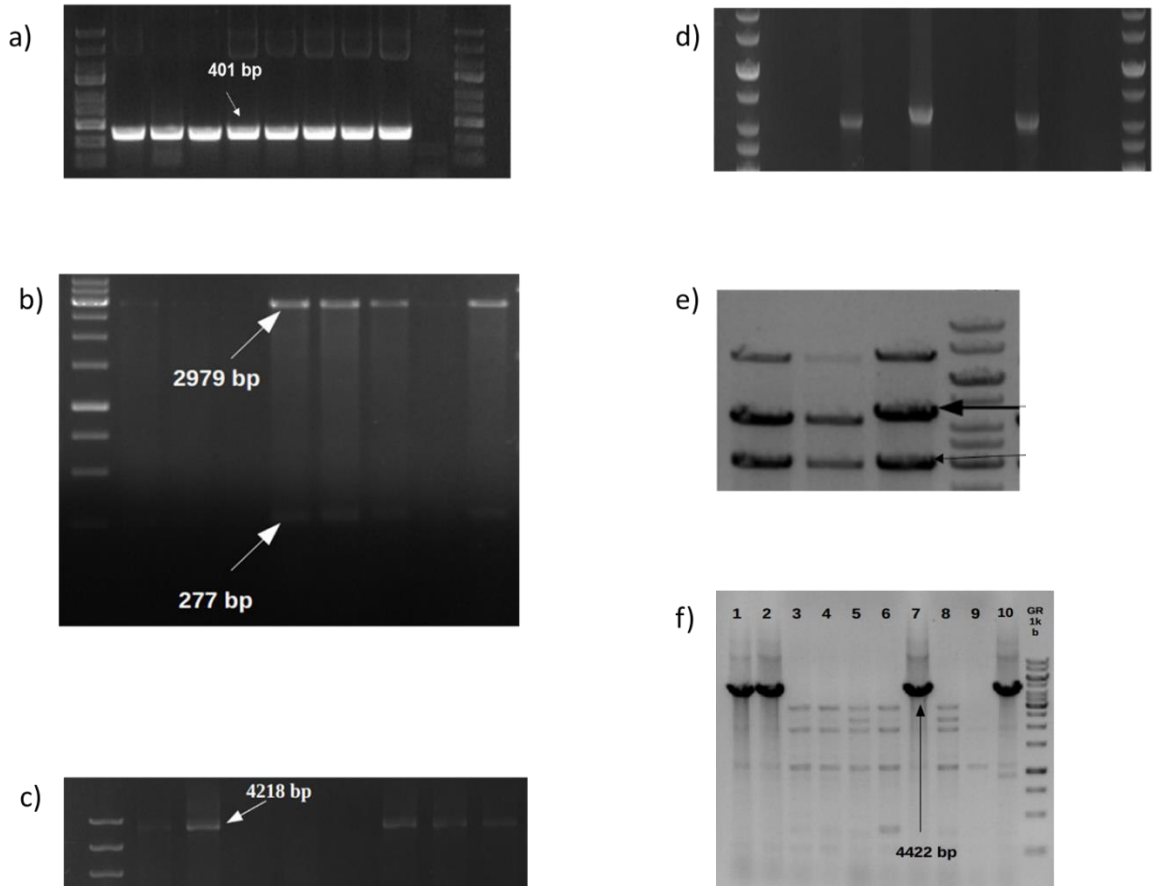

Figure 22 : Construction of psli pvmdr1, Intermidiate plasmids confirmation: **a)** Colony PCR of pjet_pfmdr1_Nterminal; **b)** Directional screening of pjet_pfmdr1_Nterminal by RFLP (AflII+ XhoI); **c)** Directional colony PCR of pjet_pvmdr1_Cterminal; **d)** Colony PCR of pjet Colony PCR of pjet_pvmdr1_full; **e)** Directional screaning of pjet_pvmdr1_full by RFLP(xbaI+XhoI) **f)** Final Plasmid (pSli-pvmdr1) confirmation: Colony PCR of pSli-pvmdr1

After transfection culture and selection of the 3D7 parasites (see figure 10), the parasitemia of the cultures were followed. Looking at the growth curves [figure 23] we can see that the transfection was a success [figure 23 a], and the pSli-pvmdr1 plasmid successfully integrated into the genome of *P. falciparum* 3D7[figure 23 b].

Never the less after running a PCR to confirm the proper integration of the plasmid [figure 24] the expected band for the proper integrant (lane 1) couldn't be observed only the *pfmdr1* was amplified (lane 2).

To understand this result, knowing that *plasmodium falciparum* 3D7 (the transfected strain) only has one copy of *pfmdr1,* a copy number variation assay was done [table 22]. The result of this test shows that the *mdr1* gene was duplicated. A hypothesis that could explain this, is that the stress generated by the prolonged drug pressured stimulated the duplication response on our parasite.

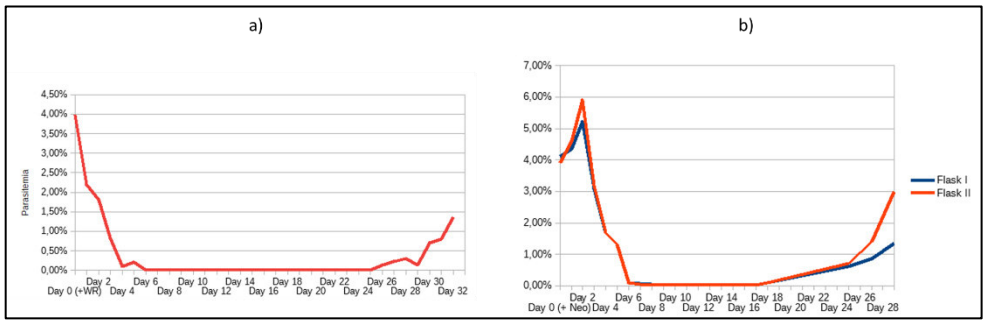

Figure 23*:* Selection of transfected *P. falciparum* with pSLI_*pvmdr1* a) selection of parasites carrying episomal plasmid with WR99210 (0,5nM) on Day 0; b) selection for integration of plasmid into genome with Neomycin 400µg/mL on Day 0,250 µg/mL on day 6

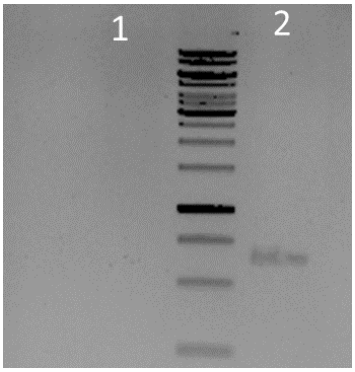

Figure 24: Integration PCR on 3D7_*pvmd1* parasites after selection. Lane 1 PCR to show proper integration of the psli_pvmdr1 plasmid; lane 2 PCR to detect *pfmdr1.*

Table 22: *pfmdr1* copy number variation on hybrid *P. falciparum* 3D7

	βtubulin		**mdr1**		**mdr1 - βtubulin**	**N-fold**
Sample	**mean CT**	**SD**	**mean CT**	**SD**		
3D7	25.91	0.38	29.97	0.15	4.06	1
3D7_*pvmd1*	22.24	0.42	25.07	0.06	2.83	2.34

5. Main conclusions

During this work the influence of both human and parasite genetic variants regarding antimaliarial treatment was evaluated. This was done first by analyzing a sample set from the Dominican Republic (DR), an endemic country for *P. falciparum* malaria. This is an interesting case study given that the DR is one of the few countries were Chloroquine is still used together with primaquine as a first line treatment for uncomplicated malaria with no evidence of clinical failure.

The first study of this work focused on the prevalence of G6PD deficiency, as the human genetic factor of interest. Because of the strong African heritage within the Dominican population, as well as the single dose of primaquine used in the country (0.75 mg/Kg), is three times higher as the dose recommended by the WHO to treat uncomplicated *P. falciparum* malaria, with no signs of complications (*i.e.* hemolytic anemia), we tested our samples for the presence of the *g6pd* 202A- mutation. Our findings showed a high prevalence of this mutation (20%). Our findings taken together with published studies by other authors suggest that the recommended doses of SLD-PQ should be higher. This result has been published by our research team in the paper "Dosage of Single Low-Dose Primaquine to Stop Malaria Transmission"[51].

The second study sougth to know, first the amount of malaria infections that can go undetected the by conventional diagnostic methods, microscopy and PfHRP2 RDTs, used in the DR by runing an ultra sensitive real time PCR detection method on samples that resulted negative for either microcipic or rapid inmuno chromatografic testing. In both cases the proportion of false negative tests was close to 20%, and in the case of RDTs.A real time PCR *pfhrp2* detection to see if deletions were responsible or not for the false negative RDTs. Our results show that for the DR, *pfhrp2* deletions are not yet a concern.

Because the Dominican Republic shares the island of Hispaniola with Haiti, its interesting to compare our results with similar studies performed in the neigbouring country. In 2015 Elbadry *et al* [102], compared the detection levels of microscopy, RDT, and PCR in 563 syptomatic Haitian patients. There PCR techqnique detected plasmodium in 19.1% of patients, as for microscopy an RDTs were only able to detect it 0.3% and 2.4% respectively. As for studies mentioning *pfhrp2* deletions in Haiti, two recent studies from 2017, and 2018 were found,[103][104]. Both studies showed that *pfhrp2* deletions have not been found west of the panama cannal ,yet metion that these mutations should be remain topics of future investigations in Haiti.

As for other *plasmodium* genetic factors, in the third study the focus were variants of the *pfcrt* and *pfmdr1* genes. Genetic markers for chloroquine resistance were looked for in the samples that resulted positive for *P. falciparum* after the real time PCR assay described in the second study. We found that in almost every instance where the *pfcrt* gene was successfully amplified, the CVIET haplotype, a very dominant CQ resistance marker was present. This study needs further confirmation through a bigger set of sampling.

The final study of this work was based on *in vitro* studies. First the calcium channel blocker verapamil was used to block the mutant PfCRT transporter carried by four isogenic *P. falciparum* strains (NF10) differing only in the *pfmdr1* N terminal polymorphisms N86Y and Y184F. Here the aim was to confirm the role of *pfmdr1* variants on anti-malarial drug resistance suggested in the previously published Veiga *et. al.* paper, "Globally prevalent PfMDR1 mutations modulate *Plasmodium falciparum* susceptibility to artemisinin-based combination therapies"[55], confirming that mutant *pfcrt* the dominant factor in conferring chloroquine resistance. Another interesting find from this study suggests that the arylaminoalcohol lumefantrine (LUM) and mefloquine (MQ) may have a different mode of action due to the differential VP response.

The second part of this study focus on repurposing a new molecular method called "selection linked integration" originally designed by Jakob Birnbaum *et al.* [74], as a tool to tag *P. falciparum* proteins and study there function, to take advantage of the fact that with this technique the selection time of recombinant parasites is significantly reduced. Here the technique was used for allelic exchange experiments using the same isogenic *P. falciparum* NF10 strains mentioned before. An attempt to replace the *pfmdr1* gene by the *P. vivax* ortholog *pvmdr1* was made. The results from this study were variable. With the Sli technique, recombination and integration of the plasmid occurred in 3 of the 5 parasites that were transfected (four *P. falciparum* NF10 and one *P. falciparum* 3D7) yet it did not happen in the predicted sites. Future work will be needed to optimize this gene editing strategy, namely for the pfcrt and for the replacement of the *pfmdr1* gene by the *P. vivax* ortholog *pvmdr1*.

6. Futures Perspectives

To better answer some the questions that arise from the molecular epidemiological part of this work in regard to the Malaria situation in the Dominican Republic a future study should be focused on working with microscopy or RDT positive samples and study the sub-microscopic cases through a much higher sample set. We aim in the future to perform a field study implementing molecular testing, using LAMP based assay, on both positive and negative microscopy or RDT.

To answer the questions regarding the high prevalence of chloroquine resistance markers and the lack of clinical failure. We first need to repeat our previous experiment this time with a more representative sample set as including samples that are positive to microscopy and /or RDTs to better determine the prevalence of chloroquine resistance markers. We also want to culture adapt parasites isolated from patients from Dominican Republic and perform full genome sequencing.

Future studies will also include in vitro drug resistance assays this time using primaquine in the same manner as we used verapamil to test this time for the synergy hypothesis.

As for the allelic exchange experiments using the Selection linked integration technique, the lack of time during the masters program did not allow for more experiments, needed to refine it. We can maybe focus on the size of the homologous region of our construct, we can also test the drug concentrations and times used during the selection process. This is valid for both experiments that employed the selection linked integration.

We still aim to modify the *pfcrt* gene in the four *P. falciparum* NF10 strains by refining the Sli technique. If the Sli system fails, we can also go back to using zink fingers nucleases, a technique that our team has used successful on previous occasions. Another option is to use the CRISPR system, that is currently being refined in the lab.

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