DISSERTATION

Submitted to the
Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University of Heidelberg, Germany
for the degree of
Doctor of Natural Sciences

presented by

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Born in Sumatera, Indonesia

Date of oral examination :_____

Aminoquinoline susceptibility of *Plasmodium falciparum* clones from Nouna, Burkina Faso, Africa

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Die vorliegende Arbeit wurde am Department für Infektiologie, Abteilung Parasitologie des Universitätsklinikum Heidelberg in der Zeit von Juli 2011 bis Februar 2018 unter der Leitung von Prof. Dr. Michael Lanzer ausgeführt.

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Datum Hani Kartini Agustar

ACKNOWLDEGMENTS

In the name of Allah, the Most Gracious and the Most Merciful. Alhamdulillah, all praises to Allah for the strengths and His blessing in completing this thesis and making this journey possible.

Firstly I would like to take this opportunity to say thank you to Prof Dr Micheal Lanzer for giving me the opportunity to work in his laboratory and pursue this project under his supervision, trust and support. The learning process and experience was enormously valuable and precious. Thank you Prof Lanzer.

Many thanks to Dr Thomas Jaenisch for accepting me as his student through skype interview conducted in 2010. After a long wait, I finally arrived in Heidelberg with high spirit and motivation to proceed my Phd degree. I am very indebted and grateful for all the helpful advices, comments and supervision during my study period. Not forgotten to Kirsten Heiss who was there always to guide and help with the 'AIberts'. I would also like to thank Christian Epp who participated in my thesis advisory committee and has introduced me to MSP1 gene. It was a very challenging task to sequence a whole gene but very fascinating. My thanks also goes to the Principal Investigators (PIs) especially Ann-Kristin, Marcel and Freddy who may have contributed ideas and suggestions through discussions in Friday seminar or during our small talks.

My special thanks to Miriam Griesheimer, for helping me with the administrative stuffs from day one until I left Heidelberg and while I am at home in Malaysia struggling to finish my writing. Your inspirational words always kept me moving and finally submitted my thesis. Thanks a million Miriam.

My heartfelt appreciation to all members of AG Lanzer for making my four years stay memorable and enjoyable experience, Cecilia, Marek, Elisa, Alessia, Sandra, Carine, Nicole, Martin, Nick, Carolin, Katharina, Sophia, Annika, Isabel, Anurag, Sebastiano, Eike, Felix, Marvin, Ines, Doro, Gabe, Tina, Costanza, Monika, and Maelle. From the department Lisi, Priyanka, Matt, Roland, Nadine, Anja, Kristin and everyone who I did not mention your name here. The list is very long. To the most important persons in the cell culture Marina and Stefan I am indebted to both of you for helping me with the cultures. The pressure you gave me made me a better parasite caretaker. I missed the atmosphere in the culture room especially during crazy times talking to the parasites.

I arrived alone to Heidelberg not knowing anyone and left Heidelberg with thousands and one friends during my stay. My special thoughts and thanks to K.Julia and Uwe Morres for my first few weeks upon arrival, same goes to Kak Zaidah and Abg Fauzi, Ikhwan and Yanti, and all my Malaysian friends who were my family

in Deutschland especially K. Azi and Akmal's family, Kak Niza and Jurgen's family, Kak Morin and Didi's family. Many thanks to Nadia, Aysun, Miriam, Gulceren, Jamila, Hatice, Mariam, Dzeneta, Serap, Seher and many more who I didn't mention here. Life was not easy but you people helped and made it very meaningful and important.

A very special note of thanks to all my colleagues in the Department of Environmental Science and Natural Resources, UKM for the help and supports in finishing my thesis especially Mardiana and Nurul Ain. Both of you have been with me from the beginning of this journey and until the end. May Allah shower His blessings upon us and hope that we bring glory and success to the faculty one day. FST to the Fore!

I would like to express my gratitude to my beloved family, Papa, Mama, my sisters Iff, Ida and Shifa, my brother in-laws, Fendi and Suhaimi, my lovely nieces, Adnin, Diana and Damiya, lastly my two adorable nephews, Solihin and Danial. Without those supports and affections I could not have made it until this far. It was a winding road and thank you so much for the trust given to me. A special thanks also to my great family in laws, Ayah, Mama Da, Aiman, Jai, Aina, Aisyah, Habib and the little ones, Nufayl and Ukayl. I am glad and happy to be a part of the family.

Finally, to my wonderful and dearest husband Hayyum. Million thanks for all the encouragement, inspiration and motivation. Despite the long distance, we are close at heart and have a unique story to tell. This journey is not perfect but passing through the path with you made it better and meaningful. You have made my life more beautiful than before. The supports and understanding has finally brought me to this final destination in my academic achievement. Thanks again for the colourful moments we have shared and for all your loves. May Allah grant us many blessings in our life.

`Anfangen ist leicht, Beharren eine Kunst´

(Starting is easy, persistence is an art)

Vielen Dank

Heidelberg

Germany

Summary

We observed a declining trend in malaria transmission at Nouna, Burkina Faso during the rainy season from 2009-2011. Single and mixed infections for *Plasmodium falciparum* and *Plasmodium malariae* were significantly reduced compared to the baseline data (rainy season 2000) and a lower prevalence of *P. malariae* was associated with a reduced transmission intensity. Microscopic examination has a lower detection limit and is associated with under-estimation of parasite burden, which suggested the use of molecular diagnosis such as PCR as a more sensitive method in determining the prevalence of malaria infections.

Amodiaquine (AQ) is an antimalarial compound chemically and functionally related to chloroquine (CQ). Currently, it is used in combination with artesunate (AS) as the first choice to treat uncomplicated *P. falciparum* malaria in some countries in Africa and South America. Chloroquine-resistant parasites and cross-resistance between CQ and AQ or its active metabolite Desethyl-amodiaquine (DQ) have been observed in Burkina Faso. *pfcrt* and *pfmdr1* are genes genetically associated with the resistance mechanism and the underlying mechanisms for cross-resistance are still under debate. The main aim of this study was to assess the susceptibility patterns of clonal field isolates *P. falciparum* compared to reference laboratory strains (Dd2 and HB3). Blood samples from 402 patients from the village Bourasso, Nouna, Burkina Faso were analysed. Genomic DNA was extracted from filter papers using the Chelex-100 method and different *Plasmodium* species were analysed by microscopy and species-specific nested-PCR.

The mutation in p*fcrt* which was associated with CQ and AQ resistance was analysed using pyrosequencing, and in vitro susceptibility of clonal parasites to CQ, AQ and DQ was determined. Three different phenotypes of *P. falciparum* (S9, S47, S173) based on the IC_{50} values were

cultured and the clonal lines were obtained. All clones from S9 and S47 harboured p*fcrt* CVIET haplotypes while all clones from S173 were CVMNK haplotype. The clones from S9 showed higher IC_{50} values on average to CQ and AQ compared to S47 clonal parasites. Some clones were sensitive to DQ for S9 and all were sensitive in clonal lines for sample 47. Clonal parasites from S173 has lower IC_{50} values towards CQ. Responses to CQ, AQ and DQ varied between the clones. Clear cross-resistance were observed in clonal lines S9, eg. 9C9, 9C7, 9H8 and in clone S47E8. There was a moderate correlation between AQ and DQ and a weaker correlation between AQ and CQ. Cross resistance exists but not high in the clonal lines.

After a consistence result of IC_{50}, 9C6, 47C7 and 173D3 were selected for the drug accumulation study. Accumulation of CQ and AQ does not always correlate with IC_{50}. It appeared that clones accumulating high levels of CQ and AQ were susceptible to CQ and AQ respectively, while clones accumulating lower CQ and AQ were resistant. High CQ and AQ IC_{50} values were associated with lower amounts of drug uptake and the two response parameters reciprocally correlated to each other. Long-term and continuous culture can cause loss of the resistance phenotype. From the observations and findings of this study, we concluded that *Pfcrt* plays a major role in the resistance to CQ and AQ and we suggest that one or more genes or SNPs may be involved in AQ drug resistance.

There were no resistant clones isolated from the *in-vitro* selection strategy after challenge with 60 nM DQ but suggested dormant parasites to DQ developed after the exposure. After investigation using quantitative recrudescence assays, the parasites were not dormant but were in fact dead. Several strategies and processes could be improved for future studies to select for AQ resistant parasites.

Zusammenfassung

Zusammenfassend haben wir eine abnehmende Tendenz der Malaria Transmission in Nouna Burkina Faso während der Regenzeit von 2009-2011 festgestellt. Einzel- und Mischinfektionen durch *P. falciparum* und *P. malariae* waren im Vergleich zu den Basisdaten (Regenzeit 2000) signifikant reduziert und ein verringertes Vorkommen von *P. malariae* stand im Zusammenhang mit einer geringeren Transmissionsintensität. Die mikroskopische Untersuchung hat ein geringeres Detektionslimit und führt zu einer Unterschätzung der tatsächlichen Parasitenbelastung. Daher wäre eine molekular-diagnostische Diagnose wie PCR eine sensitivere Methode um das Vorkommen von Malaria zu bestimmen.

Amodiaquin (AQ) ist ein Antimalariawirkstoff, welcher chemisch und funktional mit Chloroquin (CQ) verwandt ist. Aktuell ist es in einigen afrikanischen und südamerikanischen Ländern in Kombination mit Artesunat (AS) das Mittel der Wahl um unkomplizierte *P. falciparum* Malaria zu behandeln. In Burkina Faso wurden Chloroquin-resistente Parasiten sowie eine Kreuzresistenz zwischen CQ und AQ bzw. dessen aktivem Metabolit Desethyl-Amodiaquin (DQ) beobachtet. *pfcrt* und *pfmdr1* sind genetisch mit dem Resistenzmenchanismus in Verbindung stehende Gene, wobei der zugrundeliegende Mechanismus für Kreuzresistenzen weiterhin diskutiert wird.

Ein weiteres Ziel der Studie war die Suszeptibilität klonaler Parasiten von *P. falciparum* im Vergleich zu Referenz Laborstämmen (DD2 and HB3) zu bestimmen. Blutproben von 402 Patienten des Dorfes Bourasso, Nouna, Burkina Faso wurden analysiert. Genomische DNA wurde mit der Chelex-100 Methode aus Filterpapieren extrahiert und verschiedene *Plasmodium* Spezies wurden mikroskopisch sowie mittels Spezies spezifischer nested-PCR analysiert. Die mit der CQ und AQ assoziierte Mutation in *pfcrt* wurde mittels Pyrosequenzierung analysiert und die *in vitro* Suszeptibilität klonaler Parasiten gegen CQ, AQ und DQ bestimmt. Auf Grundlage der IC_{50} Werte wurden drei unterschiedliche Phänotypen von *P. falciparum* (S9, S47, S173) kultiviert und die klonalen Linien erhalten. Alle Klone von S9 und S47 enthielten den *pfcrt* CVIET Haplotyp, während alle Klone von S173 den Haplotyp CVMNK aufwiesen. Die Klone von S9 wiesen im Vergleich zu klonalen Parasiten von S47 durchschnittlich

höhere IC_{50} Werte mit CQ und AQ auf. Während nur einige Klone von S9 sensitiv gegen DQ waren, waren alle klonalen Linien von S47 sensitiv. Klonale Parasiten von S173 hatten geringere IC_{50} Werte auf CQ. Die Reaktionen auf CQ, AQ und DQ variierten zwischen den Klonen. Eine klare Kreuzresistenz wurde in den klonalen Linien von S9 z.B. 9C9, 9C7, 9C8 und im Klon S47E8 festgestellt. Es zeigte sich eine moderate Korrelation zwischen AQ und DQ sowie eine schwächere Korrelation zwischen AQ und DQ. Eine Kreuzresistenz existiert, war in den Klonalen Linien jedoch nicht stark vertreten.

Nach einem konsistenten Resultat des IC_{50} wurden 9C6, 47C7 und 173D3 für Wirkstoffakkumulationsstudien ausgewählt. Die Akkumulation von CQ und AQ korreliert nicht immer mit dem IC_{50}. Es wurde dargestellt, dass Klone welche große Mengen an CQ und AQ akkumulierten entsprechend empfindlich gegen CQ und AQ waren, während Klone welche geringere Mengen an CQ und AQ akkumulierten resistent waren. Hohe CQ und AQ IC_{50} Werte standen in Verbindung mit einer geringeren Menge an Wirkstoff und die zwei Antwortparameter korrelierten zueinander reziprok. Langzeit und Dauerkultur kann den Verlust des resistenten Phänotyps verursache. Wir schlossen aus den Beobachtungen und Ergebnissen dieser Studie, dass Pfcrt eine wesentliche Rolle bei der Resistenz gegen CQ und AQ spielt und schlagen vor, dass ein oder mehrere Gene oder SNPs in der Resistenz gegen AQ involviert sein könnten.

Die *in vitro* Selektionsstrategie resultierte nach der Exposition mit 60nM DQ nicht in resistenten Klonen sondern wies auf die Entwicklung dormanter Parasiten nach der Exposition mit DQ hin. Quantitative Rekrudeszenz Assays ergaben, dass die Parasiten nicht dormant sondern tatsächlich tot waren. Einige Strategien und Prozesse konnten für zukünftige AQ Resistenz Selektionsstudien verbessert werden.

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ABBREVIATIONS

3H	Tritium, tritiated
ACT	Artemisinin-based combination therapy
AMA1	Apical membrane antigen 1
AQ	Amodiaquine
AS	Artesunate
ATP	Adenosine triphosphate
APS	Adenosine 5'-phosphosulfate
BSA	Bovine Serum Albumin
CaCl2	Calcium Chloride
CQ	Chloroquine
CQR	Chloroquine Resistant
CQS	Chloroquine Sensitive
CRSN	Centre de Recherche de Sante' Nouna
DQ	Desethyl amodiaquine
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleoside triphosphate
EDTA	Ethylene Diaminotetraacetate
et al	*et alii* (Latin) – and others
EtBr	Ethidium Bromide
FV	Food vacuole
FVM	Food vacuolar membrane
fwd	Forward
gDNA	Genomic DNA
GmbH	Gesellschaft mit beschränkter Haftung
dH_2O	Distilled Water
H_2O_2	Hydrogen peroxide
H_3PO_4	Phosphoric Acid
HCl	Hydrochloric Acid
HEPES	N-(2-Hydroxylethyl)piperacin-N´-(2-ethylsulphonacid)
IC50	50% Inhibition Concentration

iRBC	Infected red blood cell
IS	Internal Standard
K76T	Threonine substitution to Lycine at amino acid 76
Kb	Kilobases
KCl	Potassium chloride
RON2	Rhoptry neck protein 2
rpm	rotations per minute
RPMI	Rosewell Park Memorial Institute
RT	Room Temperature
TAE	Tris/acetic acid/EDTA
Taq	Thermus aquaticus
TKM	Tris-KCl-Magnesium
TMD	Transmembrane domain
TMHMM	Transmembrane domain Hidden Markov Model
Tris	Tris (hydroxymethyl)-aminomethane
V	Volt
v/v	volume to volume
vol	Volume
w/v	weight to volume
WHO	World Health Organization
µ	Micro
M	Molar
MACS	Magnet Activated Cell Sorter
MgCl2	Magnesium Chloride
MgSO4	Magnesiumsulphate
min	Minute
ml	Milliliter
mm2	Millimeter square
mm3	Millimeter cubic
MR4	Malaria Research and Reference Reagent Resource Center
MSP	Merozoite surface protein
MQ	Mefloquine

N2	Nitrogen
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
O2	Oxygen
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Pf	Plasmodium falciparum
PfCRT	*Plasmodium falciparum* chloroquine resistant transporter (protein name)
pfcrt	*Plasmodium falciparum* chloroquine resistant transporter (gene name)
pfmdr1	*Plasmodium falciparum* multidrug resistant gene
P-gp	P-glycoprotein
pH	Potential hydrogenii
Pldh	*Plasmodium* lactose dehydrogenase
PPi	Pyprophosphate
PV	Parasitophorous Vacuole
PVM	Parasitophorous Vacuolar Membrane
QC	Quality control
QN	Quinine
R or r	Pearson regression
RBC	Red Blood Cell
RDT	Rapid diagnostic test
rev	Reverse
SP	Sulphadoxine-pyremethamine

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1.0 Introduction

Malaria has been an important disease affecting public health, for many years in both tropical and subtropical countries and is a major cause of death. Approximately, 3.3 billion people in 97 countries are at risk, leading to an approximately 200 million cases and about 600,000 deaths (WHO, 2015). Malaria is a vector-borne disease causing an infection due to a protozoan parasite, *Plasmodium*. From 120 *Plasmodium* species that infect land vertebrates, only six *Plasmodium* species are a major threat to human health (Boddey and Cowman, 2013). Of them, *Plasmodium falciparum* is one of the most important in terms of death. *Plasmodium vivax* is a major cause of illness in many regions and death due to this parasite has been underestimated (Naing et al., 2014). Nevertheless, *P. vivax* causes half of the malaria cases and is responsible for between 3.5% and 16% of the total deaths in sub-Saharan (WHO, 2015).

Furthermore, *P. vivax* is able to cause multiple relapses since it can stay dormant in infected liver cells as hypnozoites (Imwong et al., 2007). *P. ovale curtisi, P. ovale wallikeri,* and *P. malariae* are less common in causing significant disease burden. *P. ovale* also can cause relapses but there is no evidence that these parasites could generate hypnozoites (Richter et al., 2010). Nonetheless, Cogswell et al., (1991) reported hypnozoites in monkeys infected with *P. simiovale*, an analog of *P. ovale*. Another plasmodia species, *Plasmodium knowlesi*, a simian malaria parasite is an important zoonosis affecting people in Malaysia and other countries in South East Asia (Ahmed and Cox-Singh, 2015).

1.1 Malaria: Origin and history

The word malaria originates from mid 18th century Italian, *mala aria* meaning ‘bad air’, because malarial fevers were associated with swampy marshes in Italy where the disease was observed (Haldar et al., 2007).

Periodic fevers similar to malaria has been reported more than 4000 years ago. A Chinese medical document, Nei Ching from 2700 BC, mentioned the symptoms of fever were associated with splenomegaly which suggested the infection was due to *Plasmodium* parasites, the causative agent of malaria. Similarly, in the Susruta, a Sanskrit medical article, the symptoms of malarial fever were described and attributed to the bites of certain insects. During the Roman Empire and in Greece, malaria-like fever epidemics were reported to kill large proportions of the population. The first person who connected stagnant water bodies with malaria infection in the local population was Hippocrates, whose findings were later supported by the Romans (CDC, 2009).

A French army surgeon, Charles Louis Alphonse Laveran first noticed malaria parasites in 1880 in the blood of a patient who was suffering from malaria. For this discovery, he was awarded the Nobel Prize in 1907. Later in 1886, an Italian neurophysiologist, Camillo Golgi, discovered two different forms of the disease, with tertian and quartan periodicities. In 1890, Giovanni Batista Grassi and Raimondo Filetti first introduced the names *P. vivax* and *P. malariae* for two of the malaria parasites that affect humans. It was believed that there was only one parasite species according to Laveran, *Oscillaria malariae* but an American researcher, William H. Welch revised the subject again. In 1897, he named the third malaria parasite *P. falciparum*, which is widely prevalent today (Carter and Mendis, 2002) and in the same year Ronald Ross, a British officer in the Indian Medical Service, was the first to demonstrate that malaria parasites could be transmitted from infected patients to mosquitoes. Later in 1922, John William Watson Stephens described the fourth human malaria parasite *P. ovale* while the fifth malaria parasite, *P. knowlesi,* was first described by Robert Knowles and Biraj Mohan Das Gupta in 1931 in a long-tailed macaque (CDC, 2009).

The ancestors of malaria parasites possibly led a parasitic existence since there were other potential hosts to parasitize. Molecular genetic

evidence strongly suggests that this ancestor was a chloroplast-containing, free-living protozoan, which became adapted to living in the gut of a group of aquatic invertebrates (Wilson et al., 1997). The single cell organism most likely had an obligate sexual reproduction and certainly, in the present time, all Apicomplexan including malaria parasites have retained obligatory sexual reproduction in their life cycles (Kudo, 1971). The ancestors of malaria parasites were perhaps adapted to aquatic insect larvae including early Dipterans, the taxonomic order in which mosquitoes and blood-sucking flies also belong. When the first insects appeared around 150 million to 200 million years ago, certain lines of the ancestral malaria parasites attained two-host life cycles that were adapted to blood-feeding habits of the insect host.

After the presence of the early Dipterans, many different lines of malaria and malaria-like parasites evolved and radiated. Speculation regarding the evolution of *Plasmodium* species was elucidated with modern molecular techniques on genetic analysis (Fig 1.0). Based on the molecular data (Escalante et al., 1998) and biological classification (Garnham, 1996) *P. falciparum* is closely related to *P. reichenowi*, a malaria parasite infecting chimpanzees and surprisingly both are closely related to *P. gallinaceum*, a bird malaria parasite compared to other mammals. The other three human malaria parasites, *P. malariae, P. ovale* and *P. vivax* fall within a single clade that includes all mammalian malaria parasites (Escalante et al., 1998).

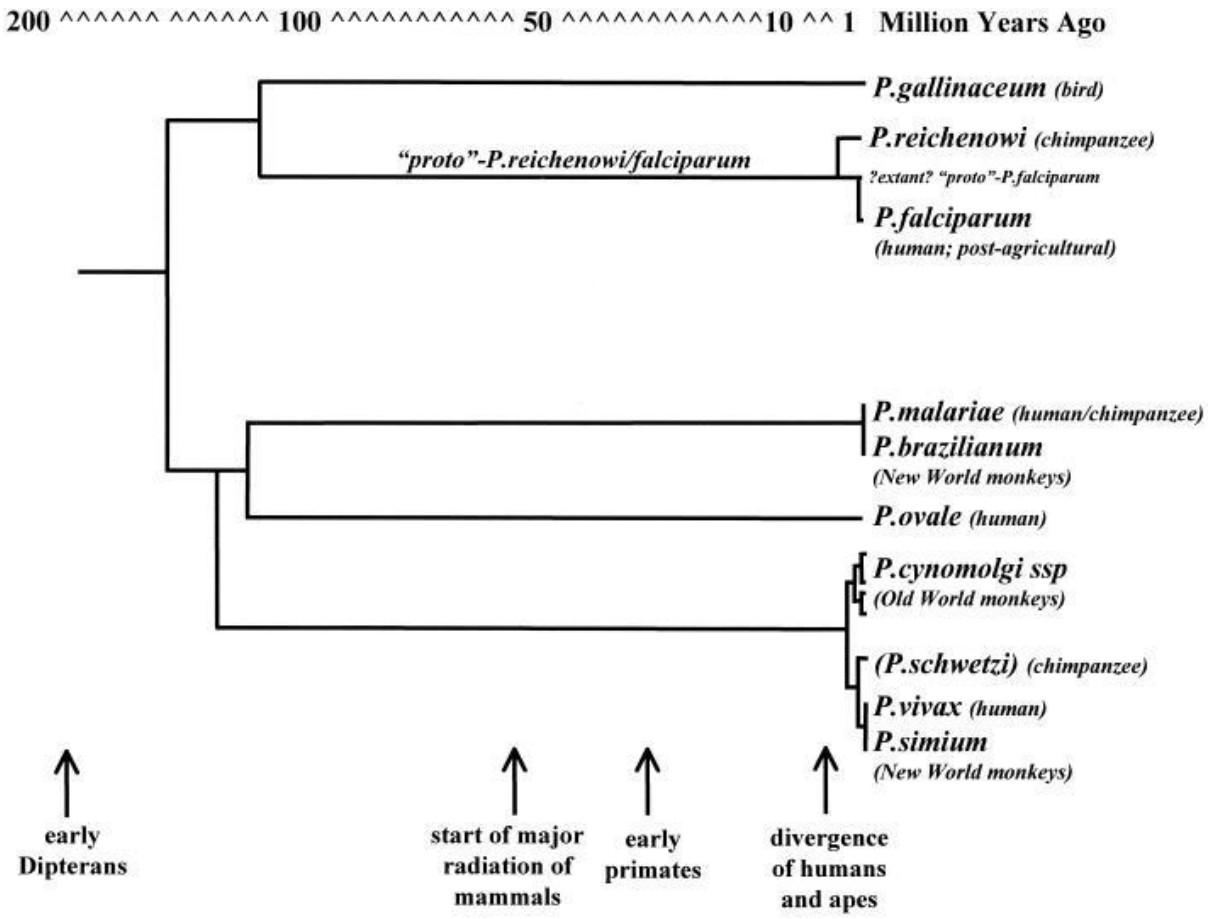

Figure 1.0 Phylogeny of malaria parasites of humans and of some other related malaria parasite species.

1.2 Epidemiology

It was estimated 216 million cases of malaria occurred worldwide in 2016 compared to 2015 with 211 million cases and in 2010 with 237 million cases (WHO, 2017). Majority of the cases in 2016 were in WHO Africa region (90%), WHO South-East Asia region (7%) and WHO Eastern Mediterranean region (2%). 15 countries, all in sub-Saharan Africa except India carried 80% of the global malaria burden.

There was a decreased from 76 to 63 cases per 1000 population at risk (estimated 18% globally) in the incidence rate of malaria between 2010

and 2016. P. *falciparum* is still the most prevalent parasite in sub-Saharan Africa while *P. vivax* is the predominant parasite in the WHO Region of the Americas representing 64% malaria cases, > 30% in WHO South East Asia and 40% in the Eastern Mediterranean regions (WHO, 2017).

In 2016, there were an estimated 445000 deaths from malaria globally compared to 446 000 estimated death in 2015. All regions recorded a decreased in mortality in 2016 when compared to 2010 except the WHO Eastern Mediterranean Region. A significant declined occurred in WHO regions of South East Asia (44%), Africa (37%) and the Americas (27%).

Countries with zero indigenous cases over at least the past three consecutive years are qualified to apply malaria free certificate status from WHO. In 2016, all countries in the WHO European Region were reported free from malaria cases. Two countries, India and Kyrgyzstan were certified malaria free in 2016 (Fig 1.1). In 2016, WHO has identified 21 countries potentially can eliminate malaria by the year 2020 which known as E-2020 countries to support their eradication acceleration targets.

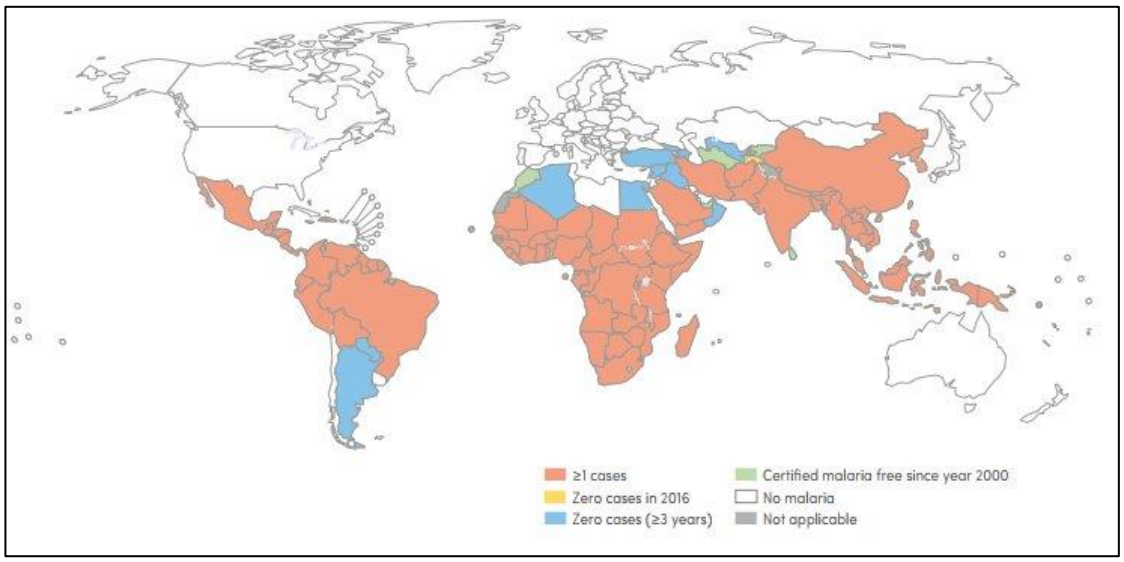

Figure 1.1 Countries and territories with indigenous cases in 2000 and their status by 2016 (WHO,2017)

1.3 Biology of Plasmodium

The protozoan parasite *Plasmodium* (Phylum: Apicomplexa, Class: Sporozoa, Order: Coccidia, Suborder: Haemosporidiae, Family: Plasmodiidae) causes an infection that is transmitted by an infectious bite of a female *Anopheles* mosquito. The life cycle of the malaria parasite is very complex and involves two hosts, a vertebrate and an invertebrate host. The infection starts when an infected *Anopheles* mosquito inoculates approximately 100 threadlike sporozoites into the skin of the mammalian host. These sporozoites are actively motile and carried by the bloodstream from the skin to various tissues and organs of the body.

The sporozoites are transported to the liver (Amino et al, 2006) where they traverse the sinusoids via Kupffer or endothelial cells and enter a hepatocyte. Active invasion is preceded by cellular traversal until a suitable hepatocyte is found. There, they form a parasitophorous vacuole membrane (PVM) and undergo asexual replication so called exo-erythrocytic schizogony until tens of thousands of daughter merozoites within 6 to 10 days, depending on the *Plasmodium* species. PVM surrounds the invading sporozoites and builds up a physical barrier between the parasite and the host (Lingelbach and Joiner, 1998). Later merozoites are released from the liver cell into the lumen of the liver sinusoids in packets of merosomes, parasite-filled vesicles (Sturm et al., 2009) into the vasculature. Once in the vasculature, the merosomes burst releasing the merozoites in the bloodstream (Prudencio et al., 2006) and begin a chronic cycle of asexual schizogony so-called intraerythrocythic schizogony. *P. vivax* and *P. ovale* furthermore develop hypnozoites in the liver, the latent forms which can stay dormant for many years until they are reactivate and cause a relapse in malaria disease (Cogswell, 1992).

In the bloodstream, merozoites bind and invade red blood cells (RBC) in a multistep processes including pre-invasion, active invasion and

echinocytosis (Weiss et al., 2015). The binding is mediated by host receptors and parasite surface protein especially the merozoite surface protein family such as merozoite surface protein 1 (MSP1). It is a major glycophosphatidylinositol (GPI)-associated protein on the merozoite surface and act as a platform on the merozoite surface (Holder, 1994). Merozoites can bind the RBC at any point on the surface but when they are attached, they reorient with the apical end pointing to the RBC. A tight junction is then formed, producing an irreversible attachment. Rhoptries secrete RON2 at tight junction and insert into the erythrocyte membrane, links the parasite with the host cell membrane through its binding to AMA1 (Richard et al., 2010)

In *P.falciparum,* the intraerythrocytic cycle lasts 48 hours and causes the clinical symptoms associated to malaria. Later, merozoite develops from ring to metabolically very active trophozoite and finally to schizont. When RBC ruptures, approximately, 10-30 merozoites are produced depending on parasite strains (Bannister et al., 2000) from each schizont then invade new erythrocytes, starting a new cycle of schizogony.

Some merozoites are reprogrammed to undergo gametocytogenesis. Gametocyte development is divided into five stages. However only stage I and stage V gametocytes are found in the bloodstream and other stages sequester in bone marrow. Within a 15-day period, once mature the gametocytes re-enter the peripheral circulation for ingestion by a mosquito where they emerge as extracellular male and female gametes in the midgut. Mating occurs by fusion of micro- and macrogametes to form a zygote that transforms over 24 hrs into an ookinete that migrates through the mosquito midgut epithelium and encysts to become an oocyst where asexual sporogenic replication occurs. Motile sporozoites are released into the hemocoel by oocyst rupture and travel to the salivary glands from where they can be injected into the next human host (Cowman, 2016).

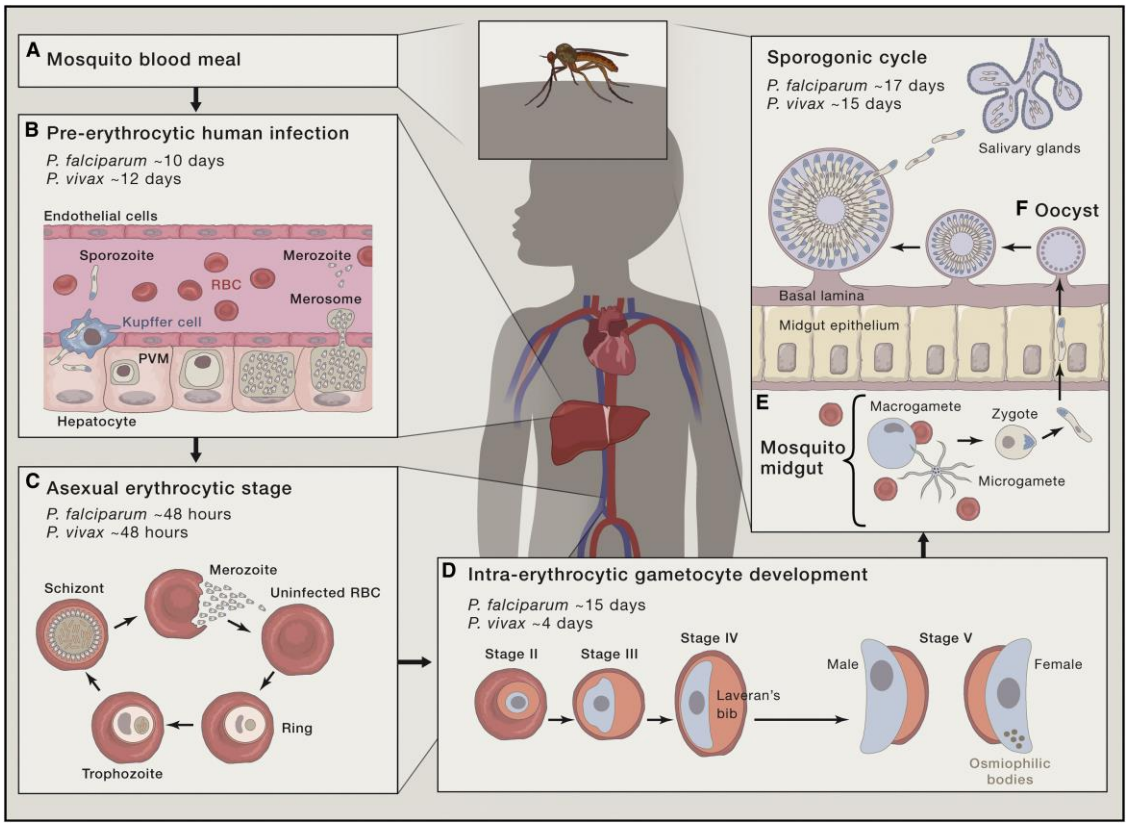

Figure 1.2 Life cycle of a Plasmodium in human and a mosquito (Cowman et al., 2016)

1.4 Clinical Manifestation

The clinical manifestations of malaria depend on several factors such as geography, epidemiology, immunity and age. In highly endemic areas, groups at highest risk include small children from 6 to 36 months who are able to develop severe illness, while pregnant women are at risk of anemia and delivering low birthweight new-borns. In areas where malaria is transmitted throughout the year, older children and adults develop partial immunity after repeated infections and are at relatively low risk for severe disease.

Unlike viral or bacterial infections, the typical symptoms of malaria are characterized by periodic fibrile episodes accompanied by chills, rigors and sweating. This is due to the release of parasite toxins into the

bloodstream during the erythrocytic cycles of merozoite egress and reinvasion of erythrocytes (Renia et al., 2016). Other common symptoms observed are headache, muscle pain, and nausea. If left untreated, uncomplicated malaria can progress to severe malaria within a few days and the outcome is 10-40% of all severe malaria cases are fatal (Schlitzer, 2007). When the disease progresses, it happens in several stages which last 6-10 hours. It started with a cold stage where patient feels cold and then followed by hot stage where fever develops along with headache and nausea. Patient also showed symptoms such as sweating and weakness. The intervals at which symptoms occur is correlated to TNF-α release from machrophages as a response to rupturing erythrocytes (Kwiatkowski et al., 1989). In the case of *P. falciparum*, parasite egress from erythrocytes is regularly unsynchronized which leads to persistent fever (Rasti et al., 2004).

Severe malaria is defined by *P. falciparum* infection, which causes dysfunction of organs, metabolic disequilibrium and the sequestration of parasites in the blood capillaries in the brain. The clinical spectrum of malaria in African children includes severe anaemia, coma and respiratory distress (Marsh et al., 1995). Anaemia is the result of erythrocyte destruction and suppression of erythropoiesis (Clark and Chaudhri, 1988) while coma happens in celebral malaria when the infected erythrocytes adhere to the brain microvasculature. Such cytoadherence is the product of PfEMP1 family proteins (Kirchgatter et al., 2005). All these pathologies can eventually lead to death (Milner et al., 2014)

Travelers visiting countries with high malaria cases generally and have no previous exposure to malaria parasites or have lost their immunity if they leave the endemic area, are at very high risk of severe disease if infected with *P. falciparum*. For this reason, it is important to consider malaria in all febrile patients with a history of travel to malaria-infected areas.

1.5 Antimalarial drugs 4-Aminoquinolines

It has been known the 4-Aminoquinolines, chloroquine (CQ) and amodiaquine (AQ), are the most important drugs to control and eliminate the infectious disease, malaria. Previously CQ was used as the first line treatment before it was replaced by sulphadoxine-pyremethamine (SP) while AQ is used as the second line treatment to treat uncomplicated malaria. However, SP was replaced by artemisinin combination therapy (ACT) in 2006 (Mohammed et al., 2013). The use of these two drugs are based on the excellent clinical efficacy, limited host toxicity, simple application and cost-effective synthesis. Generally, CQ therapy is inexpensive and practical to be used in the developing countries but has been reduced mainly as a result of the development and spread of parasite resistance.

Quinine (QN) is one of the oldest antimalarial drugs belonging to the cinchona alkaloid family. In the late 1600s, QN was first extracted from the bark of a cinchona tree. The Dutch and the British colonialists established plantations in countries, which they colonized in South-East Asia, in after the therapeutic potential of this tree was discovered. The Chinchona species is native to the Andean region of South America, but during World War II, the plantations were lost to the Japanese who initiated more research for synthetic analogues based on the QN template, such as the 4-aminoquinoline chloroquine and AQ.

Appendix 4.0 listed the antimalarial drugs used as monotherapy and summarized the quinolone derivates commonly used as antimalarial drugs along with their mechanism of action and validated molecular markers to determine their drug susceptibility or resistance.

1.5.1 Chloroquine

Chloroquine (CQ) is a 4-aminoquinoline (Foley et al., 1998). CQ is absorbed when consumed orally and available intramuscularly (IM) and intravenously (IV). The drug acts against the *Plasmodium* parasite during the asexual intra-erythrocytic phase of its lifecycle (Lehane et al., 2011). It is metabolized in the liver and mainly to monodesythylchloroquine (WHO, 2006). This drug is an amphiphilic weak base and accumulates in the parasite food vacuole because of the pH gradient.

Parasite metabolism uses its main food source, haemoglobin in the food vacuole. Heme (ferriprotoporphyrin IX), which is released as a byproduct of hemoglobin digestion, is oxidized to hematin and sequestered into hemozoin or malarial pigment by an autocatalytic mechanism (Dorn *et al*., 1995). CQ inhibits the polymerization process *in vitro* and is anticipated the same *in vivo*, causing a formation of free hematin or hematin/chloroquine complex that would finally kill the parasite (Sullivan *et al*., 1996). On the other hand, it has been suggested that weakly basic CQ accumulates to high levels in the acid food vacuole by a proton-trapping mechanism (Yayon *et al*., 1985). Therefore, CQ could lead to parasites death by direct inhibition of vacuolar enzymes such as phospholipase (Ginsburg and Geary, 1987) or proteinase (Vander Jagt *et al*., 1987).

In 1934, CQ was first synthesized and became widely used by late 1940s (Loeb et al., 1946). It has been a successful antimalarial drug due to its effectiveness, safety, low cost and antipyretic properties. Resistance to CQ emerged in the late 1950s, first in South-East Asia (Thai-Cambodian border) and in South America (Colombia and Venezuela) (Harinasuta etal., 1965, Moore et al., 1961). The spread was rapid and was also discovered in East Africa (Kenya and Tanzania) in the late 1970s (Kihamia et al., 1982, Fogh et al., 1979.) In the early 1980s, it was first reported in West Africa (Sansonetti et al., 1985, Hellen et al., 1987). Until early 2000s, although

there was relatively high prevalence of CQ resistance in Africa for more than two decades, CQ was still used and remained as the first-line treatment of uncomplicated malaria *Plasmodium falciparum.*

Regrettably, a significant increase in morbidity and mortality in children under five years was due to CQ resistance from 1980s to 1990s (Korenromp et al., 2003, Trape et al., 2001). Malawi was the first African country to change its national drug policies from CQ to SP in 1993 followed by Kenya in 1998 (Oochong et al., 2009) and Tanzania in 2001 (Taverne et al., 2001). Soon, all malaria-endemic countries in Africa stopped the use of CQ against *P. falciparum*. The change of policy to ACT as a first-line treatment for uncomplicated *P. falciparum* malaria occurred in all endemic countries between 2000 and 2009 (Gharbi et al., 2013).

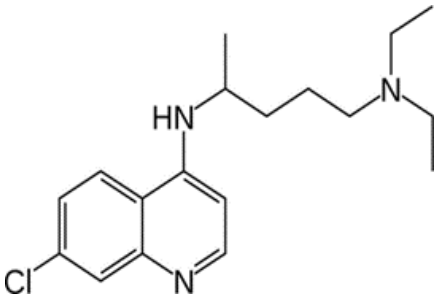

Figure 1.3 Chloroquine

Currently, molecular study has shown multiple CQR mutations and a large scale CQR selective sweep from Southeast Asia to Africa as well as other sweeps across the Amazon in South America and in PNG (Wootton et al., 2002). Wellems et al., (1990) first defined a ~400 kb DNA segment on chromosome 7 that was associated to CQR in an analysis of 16 progeny of a genetic cross of a CQ drug sensitive, HB3 and drug resistant strain, Dd2. Then the CQR locus was narrowed to a ~36 kb region using high density microsatellite markers (Su et al, 1997) which resulted in the identification of

multiple mutations in the parasite *pfcrt* gene that is associated with CQ resistance (Djimde et al., 2001) in parasites from Asia, Africa and South America (Fidock et al., 2000). The discovery of *pfcrt* , a ~3.1 kb gene with 13 exons encoding a transmembrane protein PfCRT (424 amino acid, 48.6 kDa); and multiple nucleotide substitutions in the gene were found to be linked with CQR. PfCRT is predicted to have ten transmembrane domains and is sited on the membrane of the parasite DV (Cooper et al., 2002).

1.5.2 Amodiaquine

Initially, Amodiaquine (AQ) was developed during World War II to develop substitutes for quinine by the US Army under a sponsored program (Steck, 1971). It was extensively used both prophylactically and therapeutically. AQ is a central drug in the new global strategy of combination therapies for the control of malaria (Meschnik, 2005). AQ, a 4-aminoquinoline that differs from chloroquine in having a 4- hydroxyanilino functional side chain. The medical product is known as amodiaquine hydrochloride and was first established to be effective against non-human malaria in 1946.

AQ is mainly metabolized by hepatocytes into its major active metabolite desethylamodiaquine (DQ), by the polymorphic P450 isoform CYP2C8 (Gil, 2008). The mechanism of action is thought to be similar to CQ, but it is still unclear. AQ is an alternative to CQ and is cheap and easily obtain in several countries, some with local production facilities. It is edible and easier to give to children compared to CQ. Another advantage of AQ is the long half-life of its principal active metabolite, DQ (9–18 days) (Pussard et al., 1987). In 1990, WHO suggested combination therapy of AQ particularly with artemisinin derivatives to treat uncomplicated malaria. Artemisinins are short-acting antimalarial drugs and their combination

therapy with long-acting drugs delay the development of *P. falciparum* drug resistance.

The clinical use of AQ has been severely restricted because of associations with hepatotoxicity and agranulocytosis. Due to this toxicity, WHO withdrew recommendation for the drug as a monotherapy in the early 1990s. Later, it was only reintroduced for therapeutic use. To date, there is no evidence for serious toxicity associated with AQ therapy (Olliaro and Mussano, 2003).

The AQ side chain contains a 4-aminophenol group; a structural alert for toxicity, because of metabolic oxidation to a quinoneimine. Although cross-resistance of CQ and AQ has been documented for 20 years, AQ remains an important drug due to its effectiveness against many CQ-resistant strains (Daily, 2009). Therefore, many drug design projects have since focused on reducing this toxicity.

Not much is known about the mechanism or epidemiology of amodiaquine resistance. *In vitro* studies showed that resistance to CQ and AQ are correlated; however, CQR strains appear to have lower levels of resistance to AQ (Ringwald et al., 1999). There is also evidence that AQ is effective against CQR malaria *in vivo* (Graupner et al., 2005). However, AQ resistance occurs in areas where it has been used frequently (Rwagacondo et al., 2004). More effort on this issue is clearly required.

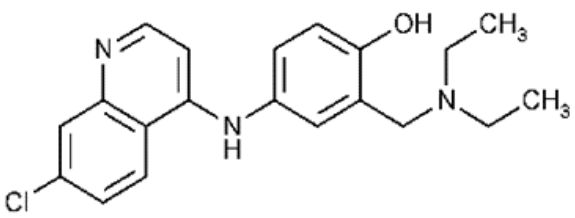

Figure 1.4 Amodiaquine

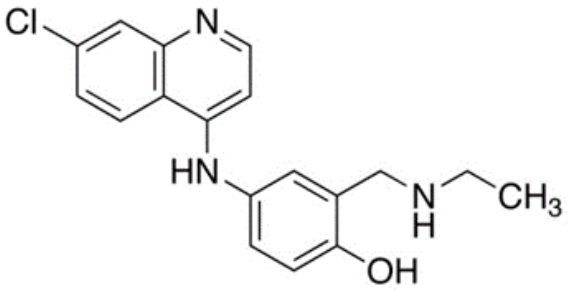

Figure 1.5 Desethyl-Amodiaquine

1.6 Genetic Markers

1.6.1. *Plasmodium falciparum* chloroquine resistance transporter (PfCRT)

The *pfcrt* gene has 13 exons and encodes a 424 amino acid transmembrane protein with a molecular mass of 48.6 kDa and localized to chromosome 7 (Fidock, 2000). The PfCRT protein belongs to the drug/metabolite transporter superfamily and chloroquine resistance transporter-like transporter family which contains 10 putative transmembrane domains spanning the digestive vacuole membrane of the parasite (Martin, 2004). Sanchez et al., (2005) reported several studies comparing the wild and mutant *pfcrt* allele expression showed less CQ accumulation when *pfcrt* was mutated as compared to wild-type *pfcrt*. The active transport mechanism of mutant PfCRT in the resistant parasite caused differences in the CQ accumulation (Sanchez, 2007). Juge et al., (2015) showed recombinant CQ-sensitive protein (PfCRT 3D7) and CQ-resistant protein (PfCRT Dd2, PfCRT 7G8, PfCRT K76T [with K76T mutation in the 3D7 gene background]) were purified and both were found to transport CQ molecule. Furthermore, higher CQ transport activity was observed with CQ-resistant protein while higher accumulation of CQ was measured with CQ-

sensitive protein by a proteoliposome study. Therefore, the CQ-resistant variants displayed decreased affinity toward CQ with increased transport activity, which lead to less accumulation of CQ in the digestive vacuole, hence conferring CQ resistance (Juge et al., 2015).

Mutations in the *pfcrt* gene plays a important role in determining CQ resistance and its phenotype. The K76T mutation is a main determinant of CQ resistance and susceptibility (Fidock et al., 2000). The K76T mutation is found in the first transmembrane domain of PfCRT protein, consists of a positively charged lysine residue which is substituted by a neutrally charged threonine residue at 76^{th} position, and permits the efflux of diprotonated CQ out of the digestive vacuole by active transport. Additional common mutations in other regions (C72S, M74I, N75E, A220S, Q271E, N326S, I356T, and R371I) also confer resistance, but only in association with K76T mutation (Martin et al., 2004).

Variation in PfCRT protein effects antimalarial drug susceptibility and resistance to other drugs such as quinine, amodiaquine (AQ), piperaquine and lumefantrine (Sisowath et al., 2009). CQ and AQ shows cross-resistance with AQ and quinine mainly mediated by 76T. The PfCRT mutations at 72-76 codons confer higher resistance to CQ and medium level AQ resistance in Southeast Asia and Africa while it is associated with greater AQ resistance in South America (Reed et al., 2000). Consequently, K76T mutation in PfCRT protein is an effective molecular marker for the antimalarial drug, subjected to the earlier use in the region.

1.6.2 *Plasmodium falciparum* multidrug resistance (PfMDR)

Another gene which is linked to CQR is the *P. falciparum* multidrug resistance protein 1 gene (*pfmdr1*). It is located on chromosome 5 consist of one exon encoding for P-glycoprotein homolog 1 (pfPgh1) protein of 1419 amino acids and 162.25 kDa molecular mass (Duraisingh et al., 2005).

PfMDR1 is a transmembrane protein with two domains, each consisting of 6 helical transmembrane domains and a nucleotide binding fold region that acts as a site for ATP binding. Similar to PfCRT, it is also exist in the digestive vacuole of the parasite and belongs to the ATP-binding cassette (ABC) superfamily. Polymorphism, amplification and variation in mRNA expression levels of the *pfmdr1* gene have been involved in resistance to numerous antimalarials and development of multi-drug resistance parasites (Duraisingh et al., 2005)

Drug susceptibility to CQ, quinine (QN), mefloquine (MQ), halofantrine, lumefantrine, and artemisinin involves mutation in the *pfmdr1* gene at the following position (N86Y, Y184F, S1034C, N1042D, and D1246Y). (Sidhu et al., 2006). PfMDR1 mutations at N86Y and N1042D positions are associated with AQ resistance. K76T and A220S mutation in the *pfcrt* gene and N86Y mutation in the p*fmdr1* gene are associated with high resistance to CQ in field isolates. In addition, copy number variation of the *pfmdr1* gene has been related to greater level of resistance to QN, MQ, halofantrine, lumefantrine and artemisinin (Sidhu et al., 2006).

1.7 *Plasmodium falciparum* drug-resistant selection

The *in-vitro* selection of *P. falciparum* drug resistant parasites was started in 1978 by Nguyen-Dinh and Trager (Nguyen, 1978) using the petri dish method. A parasite line from a Gambian (West Africa) strain (FCR3), which was resistant to 100 ng/ml CQ was generated by increasing chloroquine concentrations, starting at 10ng/ml. After 2 months (15 cycles), a parasite line that could grow in 100 ng/ml chloroquine was chosen. The resistant phenotype was stable since it could grow in drug–free medium without losing the selected features. This work has been a breakthrough, as it opened up the opportunities that a resistant strain could be chosen against an antimalarial *in vitro*.

The aim of inducing *in vitro* resistance was to generate parasite lines that can be used to investigate resistance mechanism. Many *in vitro* selection studies have been conducted such as parasite lines that were resistant against MQ in 1980s (Brockelman et al, 1981) and another selection study by Barnes et al., (1992) on chloroquine resistant parasites revealed the inverse relationship between *pfmdr1* copy numbers (mefloquine resistance) and chloroquine susceptibility. This later study initiated further investigations into *pfmdr1* polymorphism in relation to chloroquine susceptibility (Barnes et al. 1992). SNPs in this gene have been associated to chloroquine resistance and it has been recognized that *pfmdr1* plays an ancillary role in chloroquine resistance, while the primary gene is still *pfcrt* (Valderramos et al. 2006, Sanchez et al. 2007, Ekland et al. 2007).

1.8 Aims of study

The objectives of this study are as below:

- To study the prevalence of *Plasmodium* species by microscopy and PCR methods in order to perceive the variation between the dry and wet season over a period of time.
- To isolate and characterize the amodiaquine-resistant *Plasmodium falciparum* strains from Bourasso, Burkina Faso.
- To assess the *in vitro* susceptibility patterns of selected *Plasmodium falciparum* clonal parasites to a panel of Aminoquinoline drugs by using SYBR Green-fluorescent based method.
- To analyse the association between genotypic drug resistance with the phenotypic information by in vitro susceptibility testing (IC_{50}).

2.0 Materials and Methods

The underlying mechanism of AQ resistance is still not clearly understood and isolation of AQ resistant strains from field isolates might resolve as yet unanswered questions. AQ resistance may be less common than CQ resistance and cross-resistance between these drugs has been observed, suggested that the drugs share a common resistance mechanism. In this section, several methods are described detailing how the main objectives of this study were achieved.

Briefly, there were two strategies applied in isolating the AQ resistant parasites. In the first strategy, a group consisting of samples from 402 donors was chosen from October 2009 during the rainy season, confirmed positive for AQ and DQ in the serum, by the Pharmacology/Epidemiology Department. Samples identified as positive by microscopy were cultured and field isolates were then characterized by genotyping the *pfcrt* gene and performing phenotypic analysis by techniques such as in-vitro susceptibility (IC_{50}) and drug transport assays.

Secondly, a subsample that were positive by microscopy (approximately 200 samples) was chosen and challenged with the drug (60 nM DQ) as soon as the parasites appeared for two cycles. The sections below discuss each of the methods as used to accomplish the aims of this study.

2.1 Study Site

Burkina Faso is a landlocked country in Africa surrounded by six other nations (Mali, Niger, Benin, Tongo, Ghana and Ivory Coast) with the capital city Ouagadougou situated in the Province of Kadiogo in the north-west of Burkina Faso (Okrah et al., 2002). This study was conducted in a rural village of Bourasso, some 23 km from the research center of CRSN in Nouna. Nouna is the capital of the Province of Kossi and the area around Nouna town is typical of the West African savanna zone (Stich et al., 2006).

The city includes about 20,000 inhabitants in a nation with a total population of about 18.7 million. Nouna area is a dry orchard savannah, populated with farmers of many different ethnic groups. The area has a sub-Saharan climate, with a mean annual rainfall of 796 mm (range 483-1083 mm) over the past five decades. Malaria is

known to be holoendemic in this region, with prevalence highest during the rainy season; nevertheless, data on the occurrence of infection with different plasmodial species and morbidity to malaria are scarce, especially in adults. Most cases of malaria are recorded during or shortly after the rainy season, which lasts from June to of October (Muller et al., 2001).

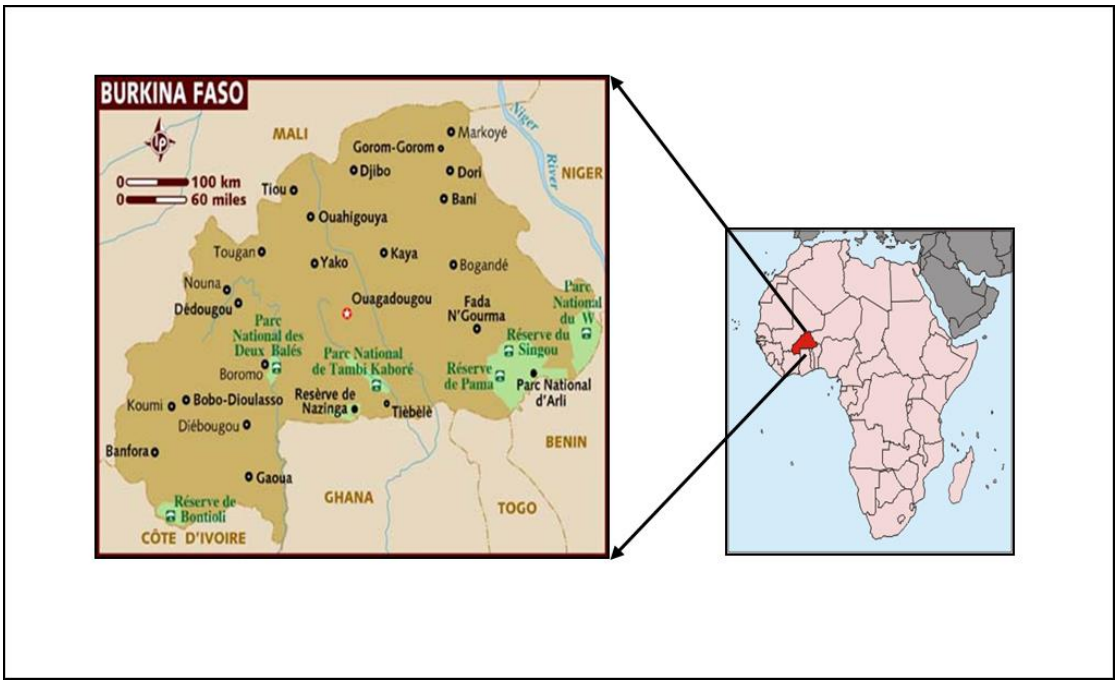

Figure 2.0 : Study site at Bourasso, Nouna, Burkina Faso

2.2 Study design and sampling

The first part of this study was designed to characterise the epidemiology of malaria in Nouna, Burkina Faso from October 2009 until April 2012, in addition to molecular markers associated with drug resistance between the wet and dry seasons. C. Geiger (2013) from AG Lanzer laboratory has conducted the first part of the study. The second part of this study was to understand the AQ resistance which has been reported in Burkina Faso since the implementation of AQ as a combination therapy with artesunate. Some parts of the epidemiological data and samples were shared and used to understand the resistance mechanism of this antimalarial drug.

A total of six sampling procedures were performed every six months from October 2009 to April 2012, of which three samples were conducted at the end of the rainy season in October and three more samples at the end of the hot dry season in April. The villagers who participated were invited based on randomized list generation to eliminate selection bias. An approval from the village council was obtained after extensive reconnaissance and written informed consent was obtained from the participants and/or parents representing their young children. Ethical approval was granted by both the ethics committee of the University Hospital Heidelberg, and by the Ethics Committee of the Centre de Recherche de Santé Nouna (CRSN).

2.3 Representatives

An article was published by Stich et al., (2006) to assess malaria endemicity in Western Burkina Faso within the entire population (n=1,561), including adults and children in year 2000. The figures and results were used as a reference study for this recent project. During the rainy season, prevalence of parasites was 80% and there was no published data for the dry season but was estimated by the team member to be approximately 15 %. A total of 1767 individuals participated in the study between October 2009 and April 2012.

	Season / No of participants	
Year	Rainy (October)	Dry (April)
2009	402	-
2010	256	362
2011	219	267
2012	-	261

Table 2.0: Number of participants per sampling from different seasons between October 2009 and April 2012

From the table above, during the rainy season (October 2009-2011), there were 402, 256 and 219 individuals respectively, while for the dry season there were 362, 267 and 261 individuals respectively. The percentage of men were slightly less than women (Table 2.1) in each sampling period. This was due to men spending more time at work outside the village than women.

Seasons	Year	Participants	Median Age(years)	Age Range (years)	Male (%)
Reference	2000	1561	14 (6-30)	0-90	50.5
Rainy (October)	2009	402	11 (5-28)	1-89	46.5
	2010	256	15 (9-37)	1-90	53.1
	2011	219	13 (6-33)	1-74	48.4
Combined data		**877**	**13 (6-31)**	**0-90**	**48.9**
Dry (April)	2010	362	14 (5-34)	1-78	44.8
	2011	267	15 (7-33)	0-77	44.9
	2012	261	13 (7-37)	1-78	45.6
Combined data		**890**	**14 (6-35)**	**0-78**	**45.0**

*modified from Geiger et al.,(2013)

Table 2.1 : Demographic data related to participants in October 2000 vs October 2009-April2012

2.4 Data and samples collection

The research team invited the participants to an evening meeting at the village Bourasso Department before the study was conducted to explain the procedures. The evening before each sampling, households were invited according to the randomized list for the next day. A random list of all households in the village was generated using the from the HDSS. Trained personnel delivered a short briefing and introduced the purpose of study and described the study design, after which participants or the parents of minor participants returned their written consent to the research team. Nurses or trained personnel recorded anthropometric data such as size and weight of the children. Temperature and respiratory rate was also recorded.

From each participant, two drops of blood were obtained from a finger prick. A blood smear was prepared and another drop of blood was dropped on a filter paper (GenoCard of Hain Lifescience, Germany or Whatman Paper 3mm chromatography filter paper by Brent Fort, United Kingdom) for further molecular analysis in the laboratory. The filter paper was dried, packed in plastic bags and dispatched to Department of Infectious Disease, Parasitology Unit Heidelberg, Germany and stored at room temperature.

Thin and thick smears were fixed with methanol and stained with Giemsa. A trained lab personnel analyzed the blood smears as per a standard protocol (Trape, 1985). The parasite density per 200 leukocytes of parasite-positive slides were determined and the parasite load per microliter by multiplying by 40. The assumed standard count was 8000 leukocytes per microliter of blood.

Each patient was examined by a physician and recent drug history was taken. All participants exhibiting the symptoms of malaria were treated with a drug combination of AQ-artesunate (AQ-AS) according to the guidelines of the Health Ministry of Burkina Faso. Additionally, trained personnel drew 5.0 ml of venous blood from positive patients and transported the blood samples to the laboratory after 2-5 hours. Serum was pipetted out under sterile condition in two aliquots and kept at -80 ° C. Serum and frozen parasites were transported on dry ice by DHL Express to Heidelberg. After the samples arrived, all were stored at -80 ° C.

2.5 Materials

2.5.1 Lab Equipments

Table 2.2 List of equipments

Equipments	Model	Company
Analytical balance		Sartorius, Göttigen, Germany
Autoclaves	ABT 120-5DM	Kern & Sohn, Balingen, Germany
	2540 EL	Tuttnauer, Breda, The Netherlands
Centrifudges	Biofuge fresco	Heraues Instruments, Hanau, Germany
Biosafety cabinet	Herasafe	Thermo Fisher Scientific, Dreieich, Germany
	SterilGrad Class II	The Baker Company, Sanford, ME, USA
	Biofuge pico	Heraues Instruments, Hanau, Germany
	Megafuge 1.0R	Heraues Instruments, Hanau, Germany
	Megafuge 1.0R	Heraues Instruments, Hanau, Germany
	Microcentrifudge 20	Hettich, Tuttlingen, Germany
Electrophoresis power supply	Power Pac 300	Bio-rad, München, Germany
	Power Pac 200	Bio-rad, München, Germany
	EPS 1001	Amersham (GE Healthcare), München, Germany

	EPS 3501	Amersham (GE Healthcare), München, Germany
Freezer -20˚C	LGex 3410 Mediline	Liebherr, Biberach, Germany
Freezer –80˚C	HERAfreeze	Thermo Fisher Scientific, Dreieich, Germany
Fridge	LKexv 3910 Mediline	Liebherr, Biberach, Germany
Heating Block	Neoblock Mono 1	Neolab, Heidelberg, Germany
Ice Machine		Ziegra, Isernhagen, Germany
Incubator	Hereaus B12/UB12	Thermo Fisher Scientific, Dreieich, Germany
Liquid Nitrogen Tank	MVE Crosystem 6000	Thermo Fisher Scientific, Dreieich, Germany
	LS 6000	Taylor-Whatson, Husum, Germany
	RS Series	Taylor-Whatson, Husum, Germany
Liquid scintillation scanner	LS6000IC	Beckman Coulter, Krefeld, Germay
Magnetic sorter	VarioMACS	Miltenyi Biotec, Bergisch Gladbach, Germany
Magnetic stirrer	RCT	IKA, Staufen, Germany
	COMBIMAG RCH	IKA, Staufen, Germany
	HR 3001	Heidolph, Schwabach, Germany
Microscope (Light)	Leica DMIL	Leica, Wetzlar, Germany
Microwave oven	R94094ST	Sharp, Hamburg, Germany
MiliQ water system	Purist Ultrapure	Rephile, Germany

Particle counter	Z1	Beckman Coulter, Krejel, Germany
pH meter	pH 7110	WTW, Weilheim, Germany
Pipetmen	P2	Gilson, Limburg an der Lahn, Germany
	P20	Gilson, Limburg an der Lahn, Germany
	P200	Gilson, Limburg an der Lahn, Germany
	P1000	Gilson, Limburg an der Lahn, Germany
Pipetus	Forty/Standard	Hirschmann, Eberstadt, Germany
Plate reader	Floustar OPTIMA	BMG Labtech, Ortenberg, Germany
PyroMark	Q96 ID	Biotage/Qiagen, Hilden, Germany
Spectrophotometer	UVIKON 923	Kontron Instruments, Munich, Germany
Puncture		Hain Lifescience, Nehren, Germany
Thermocycler	Labcycler	Sensoquest, Göttingen, Germany
	Gradient T	Biometra, Göttingen, Germany
UV Table	UV Transilluminator	Gibco BRL, Karlsruhe, Germany
Vacuum Prep Tool		Biotage/Qiagen, Hilden, Germany
Vacuum Workstation	PyroMark Q96	Qiagen, Hilden, Germany
Vortex	Genie 2	Roth, Karlsruhe, Germany
Waterbath	7A	Julabo, Seelbach, Germany

2.5.2 Software

Table 2.3 List of softwares

Software	Company
JabRef 2.10	www.jabref.org
MS Excel 2010	Microsoft Corporation, CA, USA
MS Powerpoint 2010	Microsoft Corporation, CA, USA
MS Word 2010	Microsoft Corporation, CA, USA
Sigma Plot 11	Systat Software Inc., IL, USA
Stata 11	StataCorp, TX, USA

2.5.3 Consumables

Table 2.4 List of consumables

Consumables	Company
96 well plates	Greiner Bio One, Frickenhausen, Germany
96 well microtiter plates with V-bottom	Greiner Bio One, Frickenhausen, Germany
96 well plate with fixed frame	Axon, Kaiserslautern,Germany
96 well pyrosequencing plates	Qiagen, Hilden, Germany
Accu-CheckSafe-T-Pro Plus	Roche Diagnostics, Mannheim, Germany
Adhesives film	Axon, Kaiserslautern, Germany
Aluminium foil	Carl Roth, Karlsruhe, Germany
Cryovials	Nalgene, Wiesbaden, Germany
Disposable gloves	Hartmann, Heidenheim, Germany

Disposable syringes 10 ml	Beckton Dickinson, Heidelberg, Germany
Eppendorf combi tips 2.4ml u. 0.5 ml	Eppendorf, Germany
Falcon tubes	Corning Incorporation, Bodenheim
Genocard	Hain, Lifescience, Nehren, Germany
Nitrile Disposable Gloves	Semperit, Austria
Immersion Oil	Zeiss, Jena, Germany
Slides	Marienfeld, Lauda-Königshofen, Germany
Pasteur Pipettes	Roth, Kalrsruhe, Germany
PCR tubes 0.2 ml and 8 –tube strips	Axon, Kaiserslautern, Germany
Petri dishes (10 cm diameter)	Greiner Bio-One, Frickenhausen
Petri dishes (25 cm diameter)	Greiner Bio-One, Frickenhausen
Pipette tips	Corning, Kaiserslautern, Germany
Plastic pipettes (1 ml; 2 ml; 5 ml; 10 ml; 25 ml)	Corning, Kaiserslautern, Germany
Polypropylene tubes (14 ml)	Greiner Bio-One, Frickenhausen, Germany
Whatman paper (3mm) chromatography filter paper	Brent Fort, United Kingdom

2.5.4 Biological Kits

Table 2.5 List of biological kits

Kits	**Company**
DNeasy Blood & Tissue kit	QIAGEN, Hilden, Germany
PCR Purification Kit	QIAGEN, Hilden, Germany

2.5.5 Chemicals

Most chemicals used in this study were obtained from several companies as described below:

- AppliChem GmbH
- Amersham Pharmacia Biotech Europe GmbH
- Boehringer JT Baker
- Thermo Fisher Scientific (including their trademarks Gibco, Invitrogen)
- Carl Roth GmbH
- Sigma-Aldrich (including their trademarks Fluka)
- Merck
- VWR International
- Fresenius Medical Care

Radioactive chemicals used this study were purchased from the following companies:

- GE Healthcare
  - [3H]-CQ (Reactivity :25 Ci/mmol , Conc: 1 mCi/ml)
  - [3H]-AQ (Reactiviy: 22.2 Ci/mmol, Conc: 1 mCi/ml)

2.5.6 Biological Materials

Table 2.6 List of biological materials

Biological materials	Name	Company
Enzymes	Taq DNA polymerase	Euro Clone Genomics, Italy
Marker	GeneRuler ™ 1 Kb DNA Ladder Plus	Ambion - Thermo Fisher Scientific, Dreieich, Germany

All oligonucleotides used in this study were purchased from Thermo Fisher Scientific

Table 2.7 : List of oligonucleotides

Primer	Sequence 5’ – 3’
Species –specific PCR [1]	
rPLU 6	CCTGTTGTTGCCTTAAACTTC
rPLU 5	TTAAAATTGTTGCAGTTAAAACG
FAL 1	TTAAACTGGTTTGGGAAAACCAAATATATT

FAL 2	ACACAATGAACTCAATCATGACTACCCGTC
MAL 1	ATAACATAGTTGTACGTTAAGAATAACCGC
MAL 2	AAAATTCCCATGCATAAAAAATTATACAAA
OVA 1	ATCTCTTTTGCTATTTTTTAGTATTGGAGA
OVA 2	GGAAAAGGACACATTAATTGTATCCTAGTG
Pyrosequencing PCR of Pfcrt	
Pfcrt- CF5C-Biotin	B_2 - AATTCAAGCAAAAATGACGAGCG
Pfcrt - CB10B- rev	CGGATGTTACAAAACTATAGTTACC
Pfcrt - Seq primer 76T - seq	GTTCTTTTAGCAAAAATT

[1] modified after Snounou et al., 1993

[2] B represents a biotinylation at the marked position

Table 2.8 List of parasite strains

Parasites	**Strains**	**Origin**
P. falciparum	DD2	Clonal line from Indochina (Guinet et al., 1996)
P. falciparum	HB3	Clonal line from Honduras (Bhasin and Trager, 1984)
P. falciparum	GB4	Clonal line from Ghana (Sullivan et al., 2003)

2.5.7 Buffers, Media and Solution

Table 2.9 List of buffers, media and solutions

Buffers/Media /Solutions	**Composition**
Annealing Buffer (Pyrosequencing)	20 mM Tris
	2 mM magnesium acetate
	Set pH to 7.6 with HCl and autoclave
Binding Buffer (Pyrosequencing)	10 mM Tris
	2 M NaCl
	1 mM EDTA

	Set pH to 7.6 with HCl and autoclave
	Add 0.1% Tween 20
Borate Buffer	0.2 M acid boric
	100 ml 0.1N NaOH
Complete RPMI/HEPES Medium	10% human serum
	0.2 mM hypoxanthine
	0.002% (w/v) gentamicin
	in RPMI/HEPES medium
Denaturation Solution (Pyrosequencing)	0.2 M NaOH
DNA Loading Buffer (6x)	60% glycerol
	60 mM EDTA
	0.25% Bromophenol blue
Ethidium bromide	1% solution in water (10 mg / mL) Homidiumbromid, 3,8-diamino-5-ethyl-6- Phenylphenanthridinium bromide
Freezing solution	6.2 M glycerol
	0.14 M sodium lactate
	0.5 mM KCl
	Set pH to 7.2 with NaHCO3 pH 9
	Sterilize by filtration
Glucose	360 g α- D-glucose/ L in ddH_2O 2M Sterile by filtration stored at -20 ° C
Lysis buffer for IC50	20 mM Tris-HCl pH 7.4
	5 mM EDTA
	0.008% (w/v) saponin
	0.08% (w/v) triton X-100
MACS buffer	2 mM EDTA
	1 x PBS
	Autoclave
	Add 0.5% (w/v) BSA prior to use
Mobile phase (HPLC)	ACN (15%) : TEMED Buffer (85%)
Protein lysis buffer for *P. falciparum*	0.07% (w/v) saponin in PBS

	Protease inhibitors
Sorbitol	5% (w/v) D-sorbitol in ddH_2O
	Filter sterilized
Thawing solution I	12% (w/v) NaCl
	Autoclave
Thawing solution II	1.6% (w/v) NaCl
	Autoclave
Thawing solution III	0.9% (w/v) NaCl
	0.2% (w/v) glucose
	Sterilize by filtration
TAE buffer	4 mM TRIS/acetate
	1 mM EDTA pH 8.0
TE buffer	10mM Tris pH 8.0
TEMED buffer	1.5 ml of (-N,N,N,N-Tetramethyl-ethylene diamine 900 ml dH_2O
	adjust pH to 3.5 with H_3PO_4
	Make up to 1000 mL
	1mM EDTA
TKM1 buffer	10 mM Tris pH 7.6
	10 mM Potassium Chloride
	10 mM Magnesium Chloride
	2 mM EDTA
	In 500 ml ddH_2O
TKM2 buffer	10 mM Tris pH 7.6
	10 mM potassium chloride
	10 mM magnesium chloride
	2 mM EDTA
	0.4 M sodium chloride
	In 200 ml ddH 2 O
Wash buffer (pyrosequencing)	10 mM Tris
	Set pH to 7.6 with HCl and autoclave

2.6 Methods: Culturing *P. falciparum* parasites

2.6.1 *In vitro* cultivation of laboratory strains and field isolates

Cultivation work was performed under a laminar flow (biosafety cabinet) to prevent contamination by organisms such as bacteria and yeast. Intraerythrocytic stages of *P. falciparum* were grown continuously *in vitro* as described by Trager & Jensen (1976). In this study, Dd2 (resistant) and HB3 (sensitive) strains were cultured and used as reference parasites. Regularly, all parasites were grown at 37˚C in 10 cm diameter petri dishes containing a final volume of 15ml of HEPES-buffered RPMI supplemented with 5-10% heat–inactivated A human serum (optional 5% albumax), 200 µM of hypoxanthine, 0.2 µg/ml gentamycin and 3-4% hematocrit of group A erythrocytes. Cultures were maintained under controlled atmospheric conditions: 5% O_2, 3% CO_2, 92% N_2, and 95% humidity. Parasitaemia was determined by preparing Giemsa-stained blood smear and the cultures were maintained between 1- 5 % to ensure optimal growing conditions. The cultures were monitored every 1-3 days, the medium was changed once parasitemia reached 5-10% and the cultures were split to avoid accumulation of toxic metabolites.

At the study site, we withdrew blood from participants via venipuncture, which was kept in a blood collection tube containing EDTA, an anti-coagulant to prevent blood from clotting. Blood was allowed to settle for 30 minutes to separate into plasma and cellular components. 1.0 ml was removed from the cellular phase and twice of the cellular volume of freezing solution was added.

Field isolates from the participant blood samples were cultured and grown differently. The blood group of the participants were not known, therefore, AB serum was used in the medium to avoid agglutination . A mixture of 25ml AB and 25.0 ml Albumax were added with other supplements as described. 6.0 ml of this serum was added to a 5.0 cm diameter petri dish. Each newly cultured sample was checked every 2 days and if the parasites appeared (either as rings or trophozoites) the culture was maintained until the parasitemia increased to 2-3%. Whole cultures were subsequently divided into two small 5.0 cm petri dishes and fresh medium was added to a final volume of 6.0 ml at 3.5 % hematocrit. One small culture plate was frozen

down for future used while the other plate was transferred to a 10 cm petri dish and made up to a final volume of 14.0 ml at 3.5 % hematocrit.

2.6.2 Preparation of Serum and erythrocytes

Human serum and erythrocytes were vital components in the medium used to cultivate *Plasmodium* sp. In this project, all serum and erythrocytes were received from the German Red Cross bank, Heidelberg. Human serum was aliquoted into 50 ml falcon tubes, prior incubation 800 µL of 1 M $CaCl_2$ was added and then incubated for 30 mins at 37˚C overnight. The following day, the tubes were centrifuged at 4000 rpm for 30 mins to pellet the fibrin and incubated for 30 mins at 56˚C to inactivate the serum. Later, the serum was stored at -20˚C until required. The blood received was aliquoted into 50 ml falcon tubes and 10 ml RPMI medium was added to each tube. Then, the tubes were centrifuged at 2300 rpm for 4 mins without any interruption (brake) and finally, kept at 4˚C and used within 2 weeks.

2.6.3 Freezing and thawing *P. falciparum*

Parasite cultures were frozen in liquid nitrogen in 1.5ml cryovials to store laboratory strains and field isolates for future used. Successful freezing *of P. falciparum* requires approximately 2- 5% parasitemia at the ring stage to ensure the parasites survive the freezing and thawing procedure. A culture on a 10 cm petri dish was resuspended, transferred to a 15.0 ml falcon tube and centrifuged at 1900 rpm for 2 mins at RT. The supernatant was discarded and 1/3 of the pellet volume of freezing solution was added drop by drop and mixed gently. The mixture was divided equally and distributed into two cryovials which were finally stored at -80˚C or in liquid nitrogen.

Meanwhile, when new laboratory strains or field isolates were required, a frozen sample was taken from the liquid nitrogen tank or -80˚C and warmed to RT. 200 µL of thawing solution I (12% Nacl) was added and dropped gently and transferred to a 15.0 ml falcon tube. Then, 9.0 ml of thawing solution II (1.6% NaCl) was added dropwise with a gentle shake. The solution was later centrifuged at 1900rpm for 2 mins at RT and the supernatant was discarded. Finally, 7.0 ml of thawing solution III (0.6% NaCl, 0.9% Glucose) was pipetted dropwise. The solution was later centrifuged again under the same conditions, the supernatant was removed and the pellet was

resuspended with 14.0 ml of complete RPMI medium and 0.5 ml of blood was added to the medium. The culture medium was refreshed every two days .

2.6.4 Synchronization of *P. falciparum*

Synchronization was performed when parasites at the ring stage were required for IC_{50} and drug uptake assays. The parasites were synchronized using 8.0 ml of pre-warmed 5% sorbitol as described previously by Lambros and Vanderberg (1979). The solution was incubated at 37˚C for 5 mins. After incubation, the solution was centrifuged at 1900 rpm for 2 mins and the supernatant was discarded. The pellet was resuspended again in 14.0 ml of complete RPMI medium and transferred to an empty petri dish. This sorbitol lysis technique osmotically destroys the tubule-vesicular system of trophozoites and schizonts which are absent in ring stages (Lambros & Vander Berg, 1979). To obtain a synchronous culture, the procedure was carried out in the morning and evening of the same day, and repeated after 2 days. A stable synchronous culture could only be achieved through weekly synchronization.

2.6.5 Giemsa staining and determination of parasitaemia

Approximately 50µL of blood was obtained from the bottom of the culture petri dish by using a 1.0 ml sterilized plastic pipette. A thin smear was prepared on a glass slide, air-dried for 30 sec, fixed with 100% methanol and stained with 10% Giemsa solution for 10 to 20 minutes (Fleischer, 2004). Later, the slides were washed with water and observed under an immersion oil objective with a 1000x magnification using a light microscope. The parasitaemia is defined as the percentage of parasite-infected red blood cells (counted iRBC from 1000 RBC).

2.6.6 Magnetic Cell Sorting (MACS) Purification

Purification of *P. falciparum* trophozoite and schizont stages was conducted using a MACS system. Enrichment of these stages was based on the paramagnetic properties of the hemozoin crystal in the parasite food vacuole, a product of parasite haem metabolism (Ribaut et al., 2008). Hemozoin-containing parasites were retained by metal wool in the column due to the magnetic force, while the infected red blood cells bearing ring-stage parasites were washed away.

The column was washed twice with MACS buffer and inserted into the VarioMACS separator. Then, the cultures were resuspended slowly and applied to the top of the column gradually by adjusting the stopcock to 1 drop per 3 sec. The MACS buffer was added until the flow is clear and the column was removed from the separator. The retained cells were eluted using 10 ml of MACS buffer and the eluate was centrifuged at 1900 rpm for 2 mins. Finally, the pellet was resuspended with specific buffer according to further experiments.

2.7 Other Methods

2.7.1 Determination of Amodiaquine (AQ) and Desethyl-Amodiaquine (DQ) in Serum

Chromatography was performed at ambient temperature at the Department of Pharmacology/Epidemiology, University Hospital Heidelberg. The chromatographic system consisted of a gradient pump and detector. Sample injections were performed on an injector with a 20 ul sample loop. The mobile phase consisted of Acetonitrile-TEMED buffer (15:85 [v/v] adjusted with ortho-phosphoric acid, H_3PO_4 to pH 3.5. Chromatographic separation was achieved by C_{18} column (25cm by 5µM inside diameter). The effluent column was monitored with a detector at 340 nM. The detector output was linked to a Hewlett Packard computer and software was used to analyze and record the chromatogram.

Stock solutions for AQ and DQ were prepared containing 1mg/ml of the drug and internal standard (IS) (2(7-Isoquinolinyl-ethanol) at a concentration of 1mg/ml in methanol. Working solutions at different concentrations were produced from the stock by serial dilution with dH_2O. The standard concentrations of AQ and DQ were prepared at 20, 40, 100, 250, 650, 1350 and 2000 ng/ml. Additionally for quality control, the solutions were prepared separately from the standard reference of AQ and DQ. 250 µL blank serum was spiked with the QC solutions to obtain QC sample concentrations for both drugs at 60, 775 and 1600 ng/ml. All serum samples were stored at -20˚C until analysis and thawed at room temperature before use.

Later, IS (25 µL of 0.5 µg/ml) was added to all 250 µL of thawed serum in screw-cap 15-ml polypropylene tubes, including to the standard solutions and QC samples. 0.5 ml of borate buffer at pH 10 was added to the sample mixture. The tubes and content were vortexed for 30s, then 50 ml TBME was added and fixed to a tumbling machine for 20s before centrifuging at 3000 x g for 10 mins at 15 ˚C. 45 ml of supernatant containing the organic solution was transferred to a new tube and aspirated under nitrogen gas for 25 mins at 40˚C. The residue was reconstituted with 200 µL of eluent (85% TEMED: 15% ACN) and sonicated for 2 mins, then 50 µL aliquot was injected to the HPLC system.

Calibration curves were prepared from the standard solutions (calibrators), drug-free serum spiked with the drugs to obtain concentration from 10 to 2000 ng/ml of the AQ and DQ. Freshly prepared calibrators were used during each sample analysis and the replicates were included. The calibration curve was constructed by plotting the peak area ratio of each analyte (AQ and DQ) to the IS against the corresponding concentration to evaluate the linearity (Appendix 1.0). The concentration of each drug, AQ and DQ, in the samples was calculated using the equations from the constructed calibration curve.

2.7.2 Genomic DNA (gDNA) isolation

P. falciparum genomic DNA was obtained using two protocols from the blood cultures: filter paper or Genocard. The first protocol was using DNeasy Blood and Tissue Kit (Qiagen) and the second protocol was an alcohol-based precipitation method. The methods are discussed briefly below:

Method 1 : gDNA extraction from blood culture (Alcohol-based precipitation)

The gDNA was extracted from a culture (35.0 ml, parasitemia 3-5% trophozoites, 3.5% hematocrit), resuspended and transferred into a 15.0 ml falcon tube. It was recommended to use parasites at the trophozoite and schizont stage, as these lead to greater DNA yield compared to parasites at the ring stage. The first step was to lyse the erythrocytes, each falcon tube was centrifuged at 1900 rpm at 2 mins. The supernatant was discarded and the pellet was resuspended with 15.0 ml of TKM1 buffer. 150 µL 10% of Saponin was added to lyse the erythrocyte membrane and

incubated for 5 mins on ice. Then the tubes were centrifuged at 3800 rpm for 8 mins at 4˚C in a pre-cooled centrifuged. The supernatant was decanted and pellets were redissolved again in TKM1 with equal volume as before. Finally, this was re-centrifuged as previously described and the pellet was kept at -20˚C until needed.

To obtain the gDNA, 150 µL of TKM1 was added to the frozen parasite pellet. The mixture was vortexed, then 2.4 ml of TKM2 was added followed by 150 µL 20% SDS. The mixture was vortexed again and incubated for 15 mins at 56 ˚C. The mixture was transferred to a 12.0 ml centrifuge tube (Greiner Polypropylen-12-75 centrifuge tube) and 1.35 ml 4M NaCl was added. The suspension was mixed thoroughly and then centrifuged at 11500 rpm for 10 mins at 4˚C (Beckman JA 20.1 rotor). The supernatant was collected to another new tube and two volumes of 100% ethanol was added. The tube was mixed by inverting repeatedly before it was kept for overnight at -20˚C. The next day, the tube was centrifuged at 11500 rpm for 30 mins at 4 ˚C. The pellet was obtained and washed with 500 µL of 70% ethanol and centrifuged for 5 mins. Finally, the DNA pellet was resuspended in water or TE buffer and transferred to a 1.5 ml Eppendorf tube and stored at -20˚C.

Method 2 : gDNA extraction from blood culture (DNeasy Blood Tissue Kit)

The gDNA was extracted, purified and dissolved in the same volume as in Method 1, using the DNeasy Blood Tissue Kit (Qiagen). The medium culture was divided and transferred into two different 15.0 ml tubes. Each tube was centrifuged at 1900 rpm for 2 mins. The pellet was resuspended in 10 ml of 1X cold PBS and lysed with saponin (final concentration 0.1% w/v) for 5 mins on ice. After 10 mins centrifugation at 4000 rpm at RT, the supernatant was discarded while the pellet was washed twice with cold PBS. The parasite gDNA was obtained by following the instruction of manufacturer. Briefly, the pellet was lysed with lysis buffer and proteinase K. Then 200 µL of 100% ethanol was added to precipitate the DNA. gDNA was separated from the contaminants by specific binding to silica-based membrane in high concentrations of chaotropic salt. Eventually, the samples were loaded onto a DNeasy spin column and centrifuged (8000 rpm, 1 min, RT). Washing steps were

performed twice and the gDNA was eluted with elution buffer or water and kept at -20˚C.

Method 3 : gDNA extraction from filter paper (modified from Plowe & Wellems, 1995)

Three to five punched holes of Whatman filter paper or Genocard containing blood sample were transferred to a 1.5 ml Eppendorf tube. To avoid contamination, the puncture was cleaned by pressing three times on a filter paper at a clean spot without blood sample, prior to punching new samples. Then 1.0 ml 1X PBS and 50 µL 10% Saponin were added followed by incubation overnight at 4˚C. The following day, the tubes were centrifuged at 10,000 rpm for 1 min and the supernatant was discarded. Washing was performed by the addition of 1.0 ml 1X PBS, followed by centrifugation after one hour incubation at 4˚C. The supernatant was decanted and the small punches of filter paper were soaked in 100 µL of ddH_2O.

Subsequently, 50 µL of 20% Chelex was added to the tubes and the suspension was vortexed prior to incubation at 95˚C for 10 mins. The tubes were vortexed every 2 mins. After incubation, DNA was obtained from the supernatant and purified twice by centrifugation at 13000 rpm for 10 mins. Ultimately, the supernatant was transferred to a new Eppendorf tube and the gDNA was stored at -20 ˚C until further requirements.

2.7.3 Identification of *Plasmodium* sp by PCR

The genome of *P. falciparum* contains highly polymorphic regions that may be PCR amplified and used to identify the strains (Su et al., 1999). The identification of different *Plasmodium sp* was performed based on the method described by Snounou et al., 1993. Nested PCR was applied on the different genes coding for the small ribosomal subunit RNA (ssRNA) to identify the parasite.

The PCR reaction involved several steps where initially denaturation took place between 94˚C-95˚C. Then during annealing, the temperature was lowered to allow the DNA primers to attach to the template DNA between 50˚C to 60˚C. Finally, the DNA

strand was elongated by Taq polymerase enzyme at a higher temperature and the process repeated between 25 to 40 cycles to yield higher DNA copies each time. In this experiment, the first amplification was to amplify the genus specific portion and later the fragments produced were added to three different specific primers for *P. falciparum*, *P. malariae* and *P. ovale* under the second PCR reaction. If a particular type of species was present in the sample, the primers would bind and the fragment would be amplified. In the table 2.10 below, two amplifications were carried out.

PCR 1	**(µL)**	**PCR 2**	**(µL)**
ddH_2O	17.25	ddH_2O	17.25
Euro Taq buffer (10x)	2.5	Euro Taq buffer (10x)	2.5
Magnesium Chloride (50 mM)	1.25	Magnesium Chloride (50 mM)	1.25
dNTPs (10 mM)	1.5	dNTPs (10 mM)	1.5
Primer rPLU5 (50 µM)	0.5	Primer FAL1/OVA1/MAL1 (50 µM)	0.5
Primer rPLU6 (50 µM)	0.5	Primer FAL2/OVA2/MAL2 (50 µM)	0.5
Euro Taq polymerase (5 U/µL)	0.5	Euro Taq polymerase (5 U/µL)	0.5
g DNA template	1.0	Sample from PCR1	1.0
Total	25.0		25.0

Program PCR1			**Program PCR 2**		
95˚C	5 min		95˚C	5 min	
94˚C	1 min		94˚C	1 min	
58˚C	1 min	25 cycles	58˚C	1 min	30 cycles
68˚C	1 min		68˚C	1 min	
68˚C	1 min		68˚C	1 min	
4˚C	∞		4˚C	∞	

Table 2.10 : PCR reactions and thermocycler program for parasite identification

After completion, the DNA fragments size were verified and visualized using a method called electrophoresis as described in section 2.7.3.1. Each parasite species showed a different band size: *P.falciparum* (205 bp), *P. ovale* (800 bp) and *P. malariae* (144 bp). All samples collected between the sampling period (Oct 2009 – Apr 2012)

were analyzed to investigate the prevalence of malaria at the study site and identify the *Plasmodium* species involved in the disease transmission.

2.7.3.1 Agarose Gel Electrophoresis

The amplified DNA can be separated according to the fragment size and verified by agarose gel electrophoresis. An electric field was generated in the gel matrix, causing negatively charged DNA to migrate to the anode. The agarose gel was dissolved to a final concentration of 2% (w/v) with 1X TAE buffer. Amplified DNA fragments were stained using EtBr to a final concentration of 1 ug/ml agarose and the mixture was poured onto a casting chamber with combs and left to polymerize. Intercalation of EtBr with DNA permits its visibility under UV light (254-366 nm). Prior to loading into the gel pocket, 2.0 µL of loading buffer was added to 8.0 µL of the amplified products. As a marker, the GeneRuler 1 kB plus DNA ladder was loaded in parallel with the samples. Electrophoresis was performed at a constant voltage in 1X TAE (30 ml gels maximum 90 V, 150 ml gels maximum 140 V). Finally, the gel was visualized under UV light (Gibco BRL, UV transilluminator; 260-360nm) and was photographed with a digital camera.

2.7.4 Pyrosequencing

Drug resistance is caused by point mutations in important plasmodial genes. The wild-type or sensitive strains and resistant strain parasites can be distinguished through these polymorphisms. One method to identify which allele of a marker gene a parasite is expressing is to study the specific gene fragment containing distinguishing polymorphisms.

Pyrosequencing is a DNA sequencing technique that is based on the detection of released pyrophosphate (PPi) during DNA synthesis. It has emerged as a new sequencing methodology and is widely applicable as an alternative technology for the detailed characterization of nucleic acids (Ronaghi, 2011). The technique involves four steps which includes PCR amplification of target DNA, single-stranded DNA sample preparation, single-stranded DNA template purification and nucleotide pyrosequence analysis. The first step was to amplify the target gene *pfcrt*. A part of this *pfcrt* sequence was amplified using a biotinylated primer. After amplification, single-

stranded PCR products were necessary to allow primer extension and detection of nucleotide sequence with the pyrosequencer. Several washing steps were performed to remove excess primers and salts.

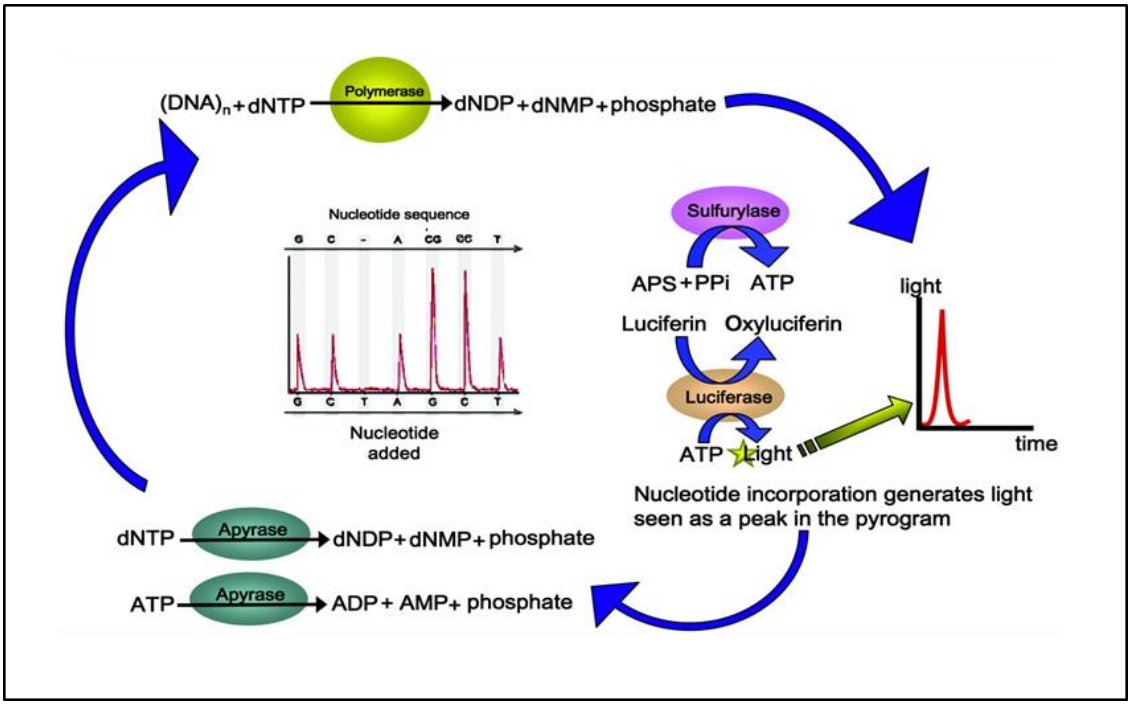

Figure 2.1: The biochemical reactions and enzymes involved in the generation of light signals by DNA pyrosequencing (Ronaghi et al., 1996). In the pyrograms, each peak represents a pulse of light detected in the instrument. The reaction involves ATP, adenosine triphosphate; ADP, adenosine diphosphate; dNDP, deoxy-nucleotidyl diphosphate; dNMP, deoxy-nucleotidyl monophosphate; PPi, pyrophosphate. Adapted from www.biotagebio.com

The chemical process involves the hybridization of a sequencing primer to the single-stranded DNA template followed by a transferred to the pyrosequencer and addition of one of four dNTPs . The reaction is catalyzed by DNA polymerase. During the process, each successful dNTP incorporation releases a PPi. ATP sulfurylase converts PPi to ATP in the presence of APS. Production of ATP drives a luciferase-mediated reaction to convert luciferin to oxyluciferin that generates visible light in amount proportional to the amount of ATP produced. The emission of light is detected by a charge-coupled device camera and represented as a peak in a pyrogram. Another

enzyme, apyrase, involved in the degradation of unincorporated dNTPs before the next dNTP is added to ensure no previously added nucleotides are incorporated into the growing DNA strand.

Selection of AQ resistance isolates was based on samples that were positive or *P. falciparum* and the serum was positive with AQ or the metabolites, DQ. Detection of SNPs that are associated with drug resistance in the *pfcrt* gene were validated by pyrosequencing. PCR reactions were performed to amplify a part of the pfcrt fragment as detailed below in Table 2.11

PCR Mix	(µL)	PCR Program		
ddH_2O	31.75	94˚C	2 mins	
Euro Taq buffer (10x)	5.0	92˚C	30 sec	
$MgCl_2$ (50 mM)	2.5	56˚C	35 sec	40 cycles
dNTPs (10 mM)	1.25	62˚C	1.0 min	
$Primer_F$ C5Fc biotinylated (5 µM)	2.0	62˚C	5.0 min	
$Primer_R$ CB10B (5 µM)	2.0	4˚C	∞	
Euro Taq Polymerase (5 U/ µL)	0.5			
gDNA	*5.0			
Total	50.0			

Table 2.11: PCR reaction to amplify the region around the SNPs in Pfcrt amino acid positions 72-76

*If the concentration of gDNA is too low the volume used was increased to 8.0 µL and adjust the ddH_20 accordingly.

** PCR Mix, program and primer varies slightly according to Zhou et al., 2006.

Hybridization of the sequencing primer was done by adding 8.0 µL of sequencing primer (Table 2.11) to 40.0 µL of annealing buffer and distributed to a PyroMark Q96 Low plate. Then, 20 µL PCR product with 3 µL Streptavidin Sepharose beads and 37.0 µL of binding to make the final volume 60.0 µL was added to form a DNA-primer mixture to Streptavidin Sepharose beads. The mixture was transferred to

a 96 well V-bottom plate (MT-plate) and kept for 5 mins at RT on the plate shaker. The plate was then heated for 30s at 80˚C. The purification of single-stranded DNA was performed by PyroMark Q96 Vacuum Prep Worktable using several solution buffers. Washing buffer, denaturation buffer and 70% ethanol were filled up at the worktable.

Then, the Vacuum Prep Tool (filter probes) was washed in the ddH_2O to check for any block filter and the Vacuum Prep Tool was lowered into the 96 well V-bottom plate to capture the beads. Later, the beads were washed with 70% Ethanol for 5s, then denaturalized by denaturation buffer for another 5s and finally washed with wash buffer for 5s. The Vacuum Prep Tool was turned upside down and allowed to dry with the vacuum switched off. Subsequently, the beads were released into the PyroMark Q96 Low plate containing 5.0 µM sequencing primer and annealing buffer with a gentle shake. Then the reagent cartridge was filled with recommended volumes of PyroMark Gold Q96 reagents (dNTPs, enzymes, and substrate) provided by the software. Ultimately, the cartridge and the PyroMark Q96 Low plate was placed in the pyrosequencer (PyroMark Q96 ID) on the dispensing unit. The set up and the analysis were conducted using the PyroMark Q96 Software v1.0.

2.7.5 Limiting dilution assay

The use of single parasite clones is a vital component to many malaria experimental studies. Techniques requiring clones include isolating parasite lines from field samples, genotyping, drug resistance testing and genetic manipulation (Liu et al., 2008). In this study, to isolate AQ resistant parasites, single clonal parasite isolates were required to ensure accuracy and unambiguous results.

Clonal parasites were obtained by limiting dilution in a V-bottom 96 well plate with an initial inoculum of 0.25 – 0.5 parasites/well. The clonal parasites can be observed visually after 2-5 weeks, either by microscopically or enzymatic detection methods (Butterworth et al., 2011). Asynchronous cultures were evaluated by microscopy to determine the parasitaemia of the culture. According to Bunn (2011), 1.0 ml of suspension at 50% haematocrit contains 5 x 10^9 of RBC, thus the total number of parasites in the starting culture can be determined from the parasitaemia and the

haematocrit of the culture. A suspension at a haematocrit of 2% in 10 ml of culture media was prepared by adding 200 µL of blood and followed by a 1:10 serial dilution in a final volume of 10 ml.

The sample tubes were gently mixed by inverting repeatedly to ensure proper mixing. To achieve a concentration cultivation of 5 parasites per mL and 2.5 parasites per mL, cultures were prepared from the serial dilution containing 50 parasites per mL in 15.0 mL medium at 2% haematocrit. 100 µL of the suspension was aliquoted into a V-bottom 96 well plate and the balance was transferred to a small petri dished as positive control. The medium was changed every 2 days. After each week, the haematocrit was increased to 1% until the third week 50 µL of the culture was removed and 50 µL of new medium at 4% haematocrit was added.

Normally, parasites appeared after two weeks in the positive control plate, followed by an increasing number of positive wells in the 96-well plates. The positive wells were indicated by the dark colour of blood, cloudy medium and confirmed by microscopy. Finally, the clonal parasites from the positive wells were transferred to a 5.0 ml petri dish to increase the number of parasites to be frozen down and for further experimention. The procedure above was repeated when 75% of the 96 well plates were positives to confirm clonal population.

2.7.6 *In vitro* drug susceptibility assays (IC_{50})

The *in vitro* drug susceptibility assay is a research tool used for several purposes such as: to monitor drug resistance, drug development screening and validate candidate molecular markers of drug resistance. There is no single, universally accepted standardized *in vitro* assay protocol. Different research laboratories take into account different factors, where each can profoundly influence the drug response. Nonetheless, it is the gold standard to measure the effectiveness of a drug by its ability to inhibit parasite growth.

In our laboratory, the method used for measuring the IC_{50} was adapted according to the standard SYBR green fluorescence-based assay (Smilkstein et al., 2004). A range of drug concentrations were chosen based both on those previously described in the literature and to ensure a sufficient number of points to determine

the IC_{50}. Two days prior to performing the experiments, the cultures were synchronized. Each sample culture was performed in triplicate to perform statistical analysis and tested for the drug studied (CQ, AQ and DeAQ). Each drug and concentration was tested in duplicate as in the scheme. The first (0% growth) and second (100% growth) column were used as controls while the last two columns were the duplicates. Parasitaemia was determined (counted iRBC from 1000 RBC) for each culture and adjusted to 0.5% parasitaemia at 3% haematocrit. The 96 well plate were prepared as in the scheme.

50 µL of medium was pipetted into each well and 25 µL was added to the first row. By using a multichannel pipette, the suspension was mixed thoroughly and then 25 µL was transferred to the second row and to the next until the last row to produce a 1:3 serial dilution. The last 25 µL was discarded. Later, 50 µL of prepared parasites were added to their respective wells to a final 1.5% haematocrit and the plates were incubated for 72 hours at 37˚C. After incubation, the plates were kept at -80˚C for at least 2 hours or until further use and thawed for 1 hour, before adding 100 µL lysis buffer containing SYBR Green (1.2 µL in 10 ml buffer) to each well. Then, the plates were shaken briefly and incubated for 1 hour at RT.

Plate scheme:

-ve	+ve	CQ_1	CQ_2	AQ_1	AQ_2	$DeAQ_1$	$DeAQ_2$	$Drug_1$	$Drug_2$	+ve	-ve
RBC*	iRBC	500 nM								iRBC	RBC*
RBC	iRBC	166.7nM								iRBC	RBC
RBC	iRBC	…	1:3 serial dilution							iRBC	RBC
RBC	iRBC	…								iRBC	RBC
RBC	iRBC	…								iRBC	RBC
RBC	iRBC	…								iRBC	RBC
RBC	iRBC	…								iRBC	RBC
RBC*	iRBC	…								iRBC	RBC*

Note :the value of RBC* usually was low and not included in the average calculation due to strong evaporation

Subsequently, the fluorescence measurements of the plates were recorded using the plate reader FLUOstar OPTIMA and the parameters were set as follows:

excitation wavelength: 485 nm; emission wavelength: 520 nm; gain: 1380; 10 flashes per well; top optic. Calculations for each fluorescence signal were performed by subtracting the background (negative control RBC) from all iRBC and tested drugs. The growth inhibition of each drug was plotted in SigmaPlot (Version 11.0) to determine the IC_{50}.

2.7.7 Drug transport assays and measurements

Clonal parasites were cultured prior to performing the experiments and synchronized using sorbitol on the previous day as described in 2.6.4. The clonal parasites were purified using a strong magnet (VarioMACS, Miltenyi Biotec, Germany), as explained in 2.6.6. This produced a purity of 98-100% trophozoite-infected erythrocytes and the concentration of parasites were adjusted to between 20000 – 30000 iRBC/µL. The exact concentration was determined by a Thoma counting chamber and later confirmed by microscopic examination of Giemsa-stained blood smears. Parasites were fed at least prior starting the purification process. Magnet-purified trophozoite-infected erythrocytes were resuspended in a pre-warmed reaction buffer (RPMI medium without bicarbonate). Then, the trophozoite cultures were incubated at 37˚C at pH7.3. The [3H]-drug (CQ or AQ/ 40 nM) was added to the preincubated culture and the time course was monitored. Every 5 min, 2 µl of 0.5 M glucose were added to the cells.

Duplicate 75 µL aliquots from each sample were transferred at various time points (5´ and 20´) to a PCR tube containing 100 µL separation oil, consisting of a mixture of 5:4 dibutylphthalate and diocytlphthalate with an equal volume of reaction buffer equilibrated at 4˚C at pH 7.3 and centrifuged at 17000 xg for 1 min. The cells were separated from the aqueous medium which contained the unincorporated [3H]-drug hence preventing further entry of the drug to the cells. Then 75 µL of the upper phase was removed from each sample and the radioactivity was determined to measure the extracellular [3H]-drug concentration. The cell pellets were accumulated at the tip of the reaction tube and recovered by cutting the tip through the oil layer using a scalpel. It was placed in a 1.5 mL Eppendorf tube and incubated overnight at

55˚C in 100 µL of tissue solubilizer (a mixture of 2:3 of ethanol and tissue solubilizer from Pharmacia).

The next day, the lysate was decolorised by the addition of 25 µL of 30% H2O2 and acidified by adding 25 µL of 1 N HCl. The lysate was transferred to a scintillation vial by cutting the lid from an Eppendorf containing 4 mL of scintillation cocktail and measuring radioactivity on a liquid scintillation counter (TRI-CARB 2100 TR, Packard). The intracellular drug concentration was calculated from the amount of labelled drug taken up by the cells, assuming the volume of a trophozoite-infected erythrocyte is 75 fL (Saliba et al., 1998). Drug accumulation was expressed as a ratio of the intracellular to the extracellular drug concentration ($drug_{in}/drug_{out}$) (Sanchez et al., 2008b).

2.7.8 Parasite screening and quantitative recrudescence assays

A group of random subsamples (n=200), which were positive by microscopy, were selected and cultured using the method described as above in section 2.6.1. The cultured parasites were challenged with 60 nM of DeAQ after the parasites appeared for two cycles. In determining the IC_{50} of CQ drugs, a cut-off value of 60 nM was used to define parasite resistance in our laboratory. Following the drug challenge test, a quantitative recrudescence assay was performed to inspect the occurrence of dormancy in AQ resistance parasites.

The quantitative recrudescence assays test was conducted based on a method described by Tucker et al., (2012). A final concentration of DQ (60 nM) was added to 15.0 ml cultures at 5% parasitaemia. After 6h post-drug exposure, the cultures were washed with RPMI 1640 to remove the drug. Prior to drug treatment, blood smears were made after 24 hours of drug exposure. Later, cultures were monitored until the normal morphological parasites appeared with 2.5% parasitaemia. Parasites were categorized as dead, dormant, ring, trophozoite or schizont. Recovery was calculated as the ratio of normal morphology parasites/total parasites while the ratio of dormant/total parasites were also calculated. Photos were taken pre-drug exposure and every 24h post-drug exposure.

2.7.9 Data and statistical analysis

The Graphs were produced using Excel 2013 and Sigma-Plot 12.5 (Systat Software, Chicago, USA). Statistical analysis was performed using IBM SPSS Statistics 25.0 and STATA 11 program (Stata Corporation, Duxbury, USA). Two-Way ANOVA with Tukey's HSD test was used to assess the significance in differences between parasite clones and drugs. Pearson's correlation was used to analyze the association between the drug susceptibilities.

3.0 Results

3.1 Prevalence of malaria cases between dry and rainy season in 2000 and 2009-2012

Malaria is holoendemic around the Nouna town area in Kossi Province of Burkina Faso, with prevalence rising during the rainy season (July-September) and the data is inadequate mainly in adults (Muller et al, 2001, 2003). One of the objectives of this study was to obtain information regarding the prevalence of malarial infections between different seasons by comparing the rainy season in 2000 as a baseline to the dry and rainy seasons from 2009 until 2012.

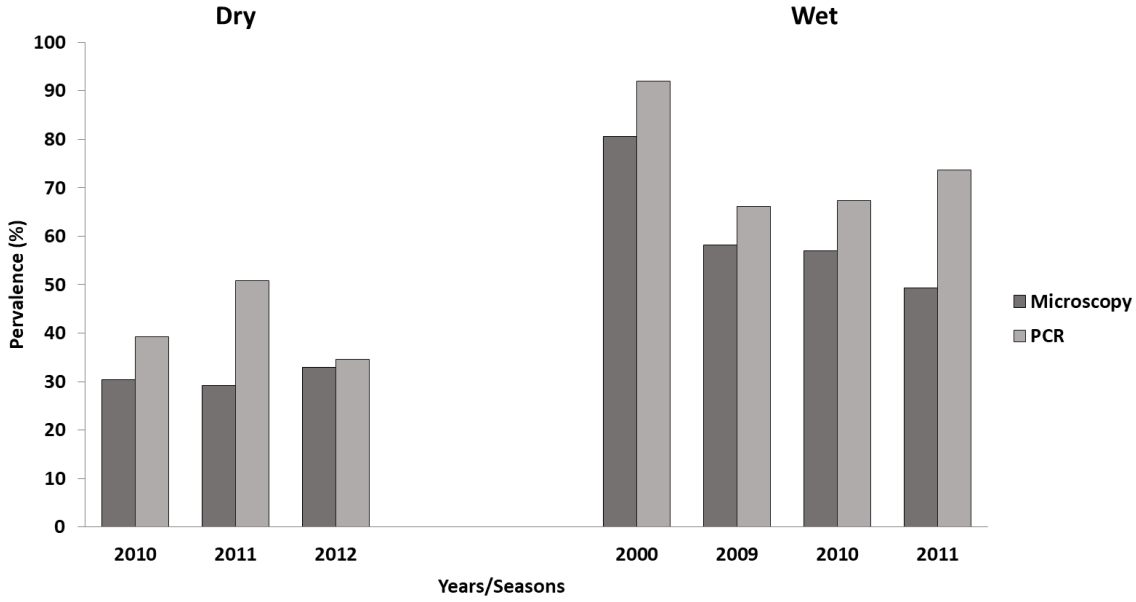

Figure 3.0 Prevalence of malaria in Nouna, Burkina Faso between dry (April) and rainy (October) season from 2000, 2009-2012. A total of 1561 residents participated in the study in 2000 during rainy season (Stich et al., 2006). The malarial infection was assessed from a random subpopulation (n=201) by microscopy, using Giemsa thick blood smear and by nested PCR from a filter paper containing blood (Snounou et al., 1993). The following study involved 1761 participants conducted during rainy season in 2009 (n=402), 2010 (n= 256) and 2011 (n=219) while in dry season 2010 (n=362), 2011 (n=267) and 2012 (n=261).

The malaria cases represented in Figure 3.0 showed a higher prevalence during the rainy season when compared to the dry season,

based on microscopy. In the dry season for April 2010, April 2011 and April 2012, the prevalence varies slightly with values of 30.4%, 29.2% and 33.0% respectively, while in the rainy season for October 2000, October 2009, October 2010 and October 2011 the prevalences were 80.6%, 58.2%, 57.0% and 49.3% respectively. The percentage of infection by microscopy decreased when compared to the reference in 2000, while the prevalence by PCR diagnosis also showed a similar trend: higher during the rainy compared to the dry season.

The PCR values were much higher compared to microscopy using Giemsa-stain. These results showed that the PCR method was more sensitive and increased the detection of prevalence to 39.3% in April 2010, 50.8% in April 2011 and 34.6% in April 2012. Similarly, in the rainy season, the occurrence of malaria cases increased to 92% in October 2000, 66.1% in October 2009, 67.4% in October 2010 and 73.7% in October 2011, as measured by PCR. The increase of prevalence in the rainy season was due to the abundance of Anopheles mosquitoes that transmit *Plasmodium* sp compared to the dry season where the abundance of vectors were lower.

The comparison between microscopy and PCR showed that a significant number of malaria cases which was also reported by Di Santi et al. (2004) and Oster et al., (2005) were undetected by microscopy. Moreover, microscopic examinations were unable to detect mixed infections and often underestimated the extent of infection, consistent with previous report (Zakeri et al. 2002). Since *P. malariae* and *P. ovale* infections are associated with long-term complications that require additional treatment, underestimating the infection could lead to a severe lapse in diagnosis. There was a significant difference

Table 3.0 Distribution of *Plasmodium* sp. during rainy and dry seasons as compared to the rainy season in 2000

Year	Seasons	Single Infection (%)			Mixed Infections (%)	
		P.f	*P. m*	*P.o*	*P.f + P.m*	*P. f + P.o*
2000	Rainy	72.0	8.3	0.7	5.1	-
2009	Rainy	50.0	0.4	4.1	1.7	-
2010	Dry	41.6	0.8	0.3	-	-
	Rainy	69.6	2.7	1.6	1.4	0.3
2011	Dry	24.0	-	-	-	-
	Rainy	40.0	2.8	-	1.9	-
2012	Dry	22.0	1.2	-	-	-

**P.f : Plasmodium falciparum P.m : Plasmodium malaria P.o : Plasmodium ovale*

Table 3.0 shows that *P. falciparum* was the most common *Plasmodium* species found in most seasons followed by *P.malariae* and *P. ovale*. In addition to single infections, the sensitivity of the PCR method allows co-infections to be detected between the seasons, especially *P. falciparum* and *P. malariae* co-infection and *P. falciparum* and *P.ovale* co-infection. In October 2010 during the rainy season, only *P. falciparum* and *P. ovale* co-infection was observed at 0.3%. During the dry season in April 2011, based on microscopy and PCR, only *P.falciparum* was identified in the samples. Another observation from the study was that the prevalence of *P. falciparum* reduced from 72% in October 2000 to 40% in October 2011 during the rainy season. Similarly, the percentage of *P. malariae* also declined from 8.3% in October 2000 to 2.8% in October 2011 during the rainy season. *P.ovale* was not detected in any samples collected in 2011 and 2012.

3.2 Amodiaquine and Desethyl-Amodiaquine concentrations

The second objective of this study was to isolate and characterize the amodiaquine-resistant *P. falciparum* strains from Bourasso, Burkina Faso. The first approach used was selecting a subsample from 402 blood samples of the rainy season (October 2009).

The concentrations of Amodiaquine (AQ) and Desethyl-amodiaquine (DQ) were determined by HPLC as described in 2.7.1. A calibration standard was prepared at 20, 40, 100, 250, 650, 1350 and 2000 ng/ml as shown in Appendix 2.0 and Appendix 3.0. The peak ratio of each analyte (AQ and DQ) to the IS was plotted against the corresponding concentration to make calibration curves and determine the linearity of the method on each day of analysis.

Regression analysis was performed using Microsoft Excel 2013 to determine the slope, intercept and the correlation coefficient (r) of each calibration curve. The concentrations of analytes were calculated using the equation obtained from the calibration curves. The method was validated by adding three Quality Controls (QC) with different concentrations that included the low, medium and upper range of the calibration curves. Furthermore, 25.0 ul of an Internal Standard (IS) (1mg/ml in methanol) was also added to each sample including standards and QC in order to ensure reliable quality and consistent results.

A total of 42 serums were positive for either AQ or DQ. Of the 42 AQ or DQ positive samples, only 15 samples that were positive by microscopy or PCR, were selected for further analysis. Blood samples from 15 identified patients were cultured as described in 2.6.1 for *in vitro* culture of the laboratory strains and field isolates. Three laboratory strains with different phenotypes (HB3, DD2 and GB4) were used as reference strains in search for the AQ-resistant isolates from Burkina Faso. HB3 is a chloroquine sensitive

strain while DD2 and GB4 are chloroquine resistant strains. The cultures were maintained cautiously to prevent bacterial or fungal contamination.

Table 3.1 Concentration of Amodiaquine (AQ) and Desethhyl-Amodiaquine (DQ) (ng/ml) in blood samples that were positive by microscopy.

Lab strains/ Field isolates	**[AQ] /[DQ]/ng/mL in patient's blood**
S47	<LOQ
S6	38.85
S9	32.5
S79	34.29 (AQ)
S166	50.69
S173	LOQ
S256	LOQ
S278	48.03

** < Limit of Quantification(LOQ) = < 24 ng/mL

Table 3.1 Concentrations of AQ and its metabolite DQ determined from each blood sample. From 15 cultured isolates, only eight could be rescued and adapted to laboratory conditions. The number denotes the patients ID during the rainy season in October 2009. AQ was detected in sample 79 (S79) at 34.29 ng/ml. In the other blood samples, concentration of desethyl-amodiaquine was measured at 38.85 ng/ml, 32.50 ng/ml, 50.69 ng/ml and 48.03 ng/ml for S6, S9, S166 and S278 respectively. S47 has detected DQ below the LOQ and also S173 and S256, DQ was detected at LOQ which was at 24 ng/ml. LOQ were determined by signal-to-noise (S/N) ratio evaluations of analytes. It is defined as the lowest concentration of analyte with S/N ratio at least 10 and acceptable accuracy and precision (15%) (Braggio et al. 1996).

3.3 Single Nucleotide Polymorphisms (SNP) in the PfCRT haplotype of *P. falciparum*

Further investigation was performed to characterize the eight laboratory-adapted field isolates. To amplify the pfcrt region, DNA was isolated from each strain and used for pyrosequencing. The primers CF5C and CB10B was used to amplify a region of pfcrt which includes the polymorphic region in exon 2. CB10B was biotinylated to detect polymorphisms in codons corresponding to amino acid 72 while CF5C was biotinylated to detect polymorphisms at amino acid position 76. The technique involved the quantification of a light reaction which depends on the amount of nucleotide fused at a specific base pair position and allows the allele to be determined. Mutations between acid amino acids 72 and 76 in the polymorphic region are characterized by the haplotype CVMNK (wild-type) and haplotype CVIET (resistant-type) with three modified bases 'IET'. Below, Figure 3.1 presents examples of pyrograms produced after each DNA sample was analysed by this technique.

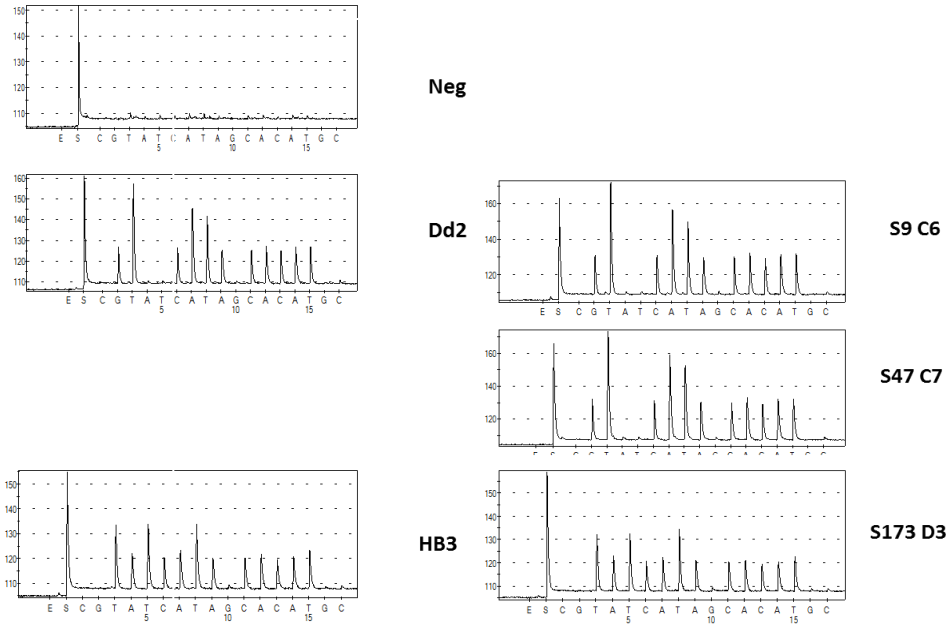

Figure 3.1 Selected Pyrograms for pfcrt genotyping which includes DD2 and HB3 as reference while S9C6, S47C7 and S173D3 are the clonal lines. The haplotype was determined by reading the pyrogram from right to left. X-axis represents the nucleotides while Y-axis represents the light intensity. Height ratio referred to number of nucleotides for eg. the reference resistant strain DD2 consist CVIET haplotype while the reference sensitive strain, HB3 consist of CVMNK haplotype.

Table 3.2 *pfcrt* haplotypes for eight cultured isolates (parental clones) which were positive for AQ in the serum including three reference strains HB3, Dd2 and GB4.

	Positions within *pfcrt*				
Lab strains/ Parental clones	**72**	**73**	**74**	**75**	**76**
S47	C	V	I	E	T
S6	C	V	I	E	T
S9	C	V	I	E	T
S79	C	V	I	E	T
S166	C	V	I	E	T
S173	C	V	M	N	K
S256	C	V	I	E	T
S278	C	V	I	E	T
HB3	C	V	M	N	K
Dd2	C	V	I	E	T
GB4	C	V	I	E	T

Table 3.3 Single nucleotide polymorphisms (SNP) analysis for clonal lines

Clones S173	PfCRT Haplotype	Clones S9	Clones S47	PfCRT Haplotype
S173 A1	CVMNK	9A4	S47 C1	CVIET
S173 A11	CVMNK	9 A6	S47 C7	CVIET
S173 A12	CVMNK	9 C6	S47 D4	CVIET
S173 B11	CVMNK	9 C9	S47 D7	CVIET
S173 C7	CVMNK	9 D7	S47 E8	CVIET
S173 D3	CVMNK	9 E3	S47 F8	CVIET
S173 D10	CVMNK	9 F2	S47 G6	CVIET
S173 E8	CVMNK	9 F11	S47 G8	CVIET
S173 G6	CVMNK	9 H7	S47 H8	CVIET
S173 H8	CVMNK	9 H8	S47 H10	CVIET

Based on Table 3.2 and Table 3.3, genotypic analysis allowed the determination of the *pfcrt* haplotype within the parental clones and clonal lines. The results show that all parental clones harbour the PfCRT haplotype CVIET except sample no. S173. No other PfCRT haplotype such as SVMNT, which is more prevalent in South America, Afghanistan, Iran, Laos (Sa et al. 2009) was detected during the genotypic analysis.

Three parental clones from the drug susceptibility assay in Figure 3.2 were selected. The selection was based on different drug susceptibilities and performed in parallel with the genotyping. After limiting dilution, individual clones were obtained and a similar genotypic analysis conducted after which ten clones were selected from each parental line. All clonal lines from sample S173 has a similar PfCRT haplotype as the sensitive strain HB3 (CVMNK), while clonal lines from S9 and S47 were comparable to the resistant strain Dd2, which harbours the CVIET haplotype.

3.4 Characterization of field isolates

There are several ways to evaluate drug resistance. *In vitro* testing of *P.falciparum* is one of the most important tools to monitor the efficacy of anti-malarial drugs (Briolant et al., 2009). In this study, field isolates were cultured according to the method developed by Trager and Jensen approximately 40 years ago (Trager, 1976). Parasite susceptibility to CQ, AQ and its metabolites, DQ were measured using Syber Green, a fluorescent dye-based *in vitro* drug sensitivity assay. The eight field isolates which were serum positive for AQ or DQ were tested for resistance to AQ and CQ.

There are wide variations in the threshold used to define *in vitro* resistance of *P. falciparum* to amodiaquine, probably due to the differences in protocols and interpretation (Echeverry, 2006). In our laboratory, the threshold for CQ resistant strains was estimated at approximately ≥60 nM while that for AQ resistant strains was estimated to be ≥ 20 nM. The *in vitro* susceptibility of the isolates was determined using IC_{99}, IC_{90} and IC_{50}. (IC: inhibitory concentration). IC_{50} is widely used and defined as the concentration of the drug that inhibits the growth of 50% of the parasites as compared to the control without drug (Echeverry, 2006). The values were determined by non-linear regression analysis in which the logarithm of concentrations was plotted against growth inhibition. A sigmoid curve was fitted to the plot using the Sigmaplot 12.5 software to obtain IC_{50} values.

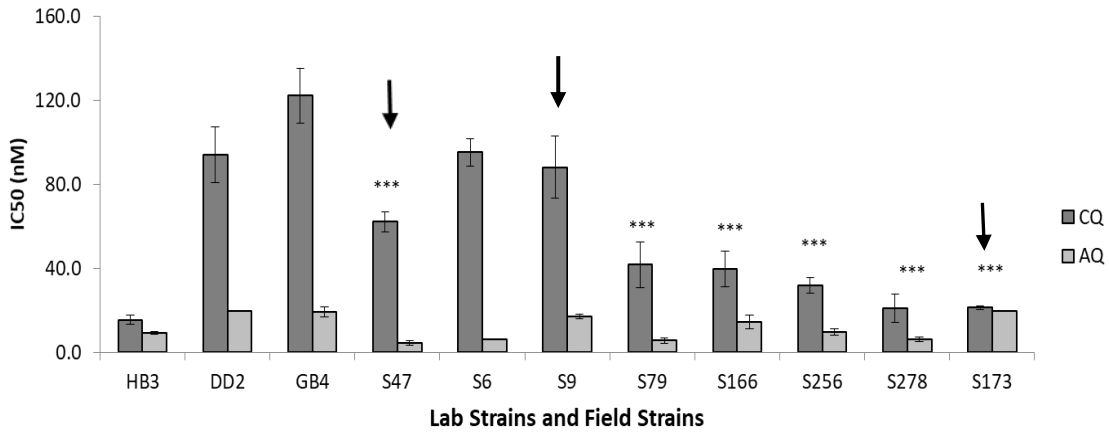

*Figure 3.2 Drug susceptibility of the reference strain DD2, GB4 and HB3 towards CQ and AQ, together with the IC_{50} values of selected field isolates which successfully cultured in the laboratory condition. The data represent the mean±SEM of three to five independent determinations per isolates. Arrow showed the three isolates with different phenotype (susceptibility) chosen for further investigation. Two-Way ANOVA test was performed to check for differences between isolates and drug at level ***=p <0.05*

Figure 3.2 shows the results of IC_{50} values for eight field isolates with three reference strains (HB3, DD2 and GB4). The field samples harboured CQ sensitive and resistance strains when compared to the IC_{50} reference value given by HB3 (15.47±2.16) nM, Dd2 (94.26±13.23)nM and GB4 (122.30±13.04) nM. Similarly, the AQ sensitivity values range from 4.40±1.09 to 9.74±1.49 nM while for AQ resistance, the values range from 14.41±3.34 nM to 19.58±0.32 nM. Three field isolates, which were S6, S9 and S47, showed resistance to CQ while S79, S166, S173, S256 and S278 the IC_{50} values where lower than 60 nM indicating that these samples were sensitive to CQ.

For the AQ drug *in vitro* assay, the values were 16.98nM and 19.58nM for S9 and S173 respectively indicating resistance to AQ while the all other field isolates showed values below the threshold <20 nM and were therefore considered sensitive. Comparatively, to reference strain HB3 which has the value of 9.21nM for AQ sensitive strain. Sample S9, S47 and

S173 were chosen to obtain clonal parasite lines by limiting dilution. S9 exhibited resistance towards CQ and AQ while S47 demonstrated an intermediate resistance towards CQ and AQ. Lastly, S173 was sensitive to CQ and resistant to AQ. S9 was cultivated from a male child's blood (4 years old) whose serum tested positive for DQ at 87.8 nM. Then, S47 was obtained from a female child of 2 years while S173 was also taken from a male child of 3 years of age. For both samples S47 and S173, the concentration of DQ was identified below the LOQ which was 65.8 nM.

3.4.1 Susceptibility patterns of clonal parasites

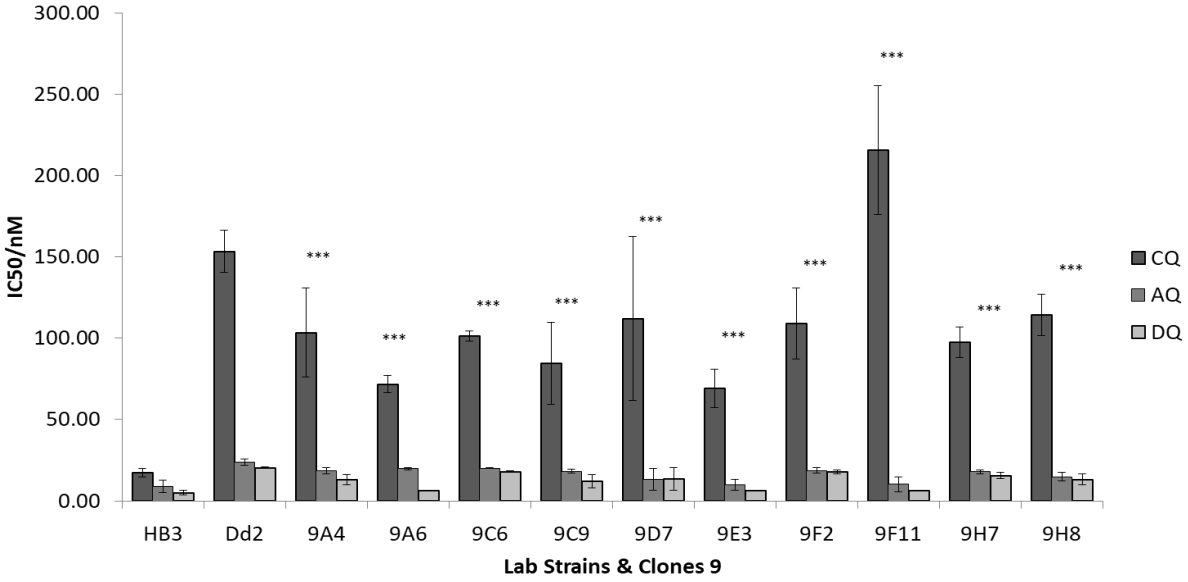

*Figure 3.3 Drug susceptibility of the S9 clonal lines towards CQ, AQ and DQ, including the reference HB3, Dd2 and GB4. Ten clones of S9 were chosen from a limiting dilution assay and the IC_{50} values were obtained using the SYBR Green method. In brief, after 72 hours incubation, the test plate was removed and frozen down. Before measuring, the plates were thawed and 100 uL of lysis buffer containing SYBR Green fluorescent dye was added to each well and mix thoroughly. Then, the plate was incubated in the dark for at least one hour. The data represent the mean±SEM of at least three to five independent determinations per clones. Two Way ANOVA test was performed for significance difference between isolates and drug at level *** = p<0.001*

The above figure shows the drug response for CQ, AQ and DQ. Ten clones were chosen from the parental clone S9. All clones were resistant to CQ when compared to the reference strain DD2. The value of the observed IC_{50} ranged from 71.8 to 215.5nM, while the IC_{50} value for AQ were recorded from 9.8 to 20.1 nM. For DQ the readings ranged from 6.25 to 18.0 nM for the selected clones. Most clones of S9 were sensitive to DQ. The readings recorded for each drug response between clones varies due to the difference in genetic background of each clone. The highest IC_{50} for CQ was shown by clone F911 (215.45 ± 39.72 nM), while for AQ the highest value was observed for clone 9C6 (20.10 ± 0.21 nM) whereas for DQ the values were quite low compared to the Dd2 reference strain which was recorded at

17.97 ± 0.58 nM. A significant variation was observed between the drugs and the clones using a Two Ways Anova (***= $p<0.001$). Between drugs, CQ and AQ were statistically different and also between CQ and DQ at level $p<0.001$. A clear cross-resistance was observed in clone 9C6, 9F2 and 9H8.

The aim of this investigation was to obtain one clone that was highly resistant to CQ, AQ and DQ. Therefore, based on the above results, only one clone was selected for sample S9 which featured high drug susceptibiliy to CQ, AQ and DQ. Although 9C6 (CQ: 101.18 ± 3.19 nM, AQ: 20.10 ± 0.21 nM, DQ: 19.97±0.58 nM) and 9F2 (CQ: 109.00 ± 22.02 nM, AQ: 18.73 ± 1.69 nM, DQ: 17.73 ± 1.21 nM) clones represents the closest features required only 9C6 was selected because the variation in IC_{50} for CQ was lower compared to clone 9F2. Referring to Table 3.3, all clones were genotypically resistant since they harboured the *pfcrt* CVIET haplotype. In spite of the haplotype resistance, some clones showed a high response to CQ and AQ while some showed lower IC_{50} values. Surprisingly, only two clones, 9C6 and 9F2 have IC_{50} values of AQ and DQ, which were closely resistant compared to Dd2. The confirmation of the resistance phenotype will be studied further in the following drug accumulation test. Based on this study, clone 9C6 was chosen for further investigations since it was resistant to CQ, AQ and DQ.

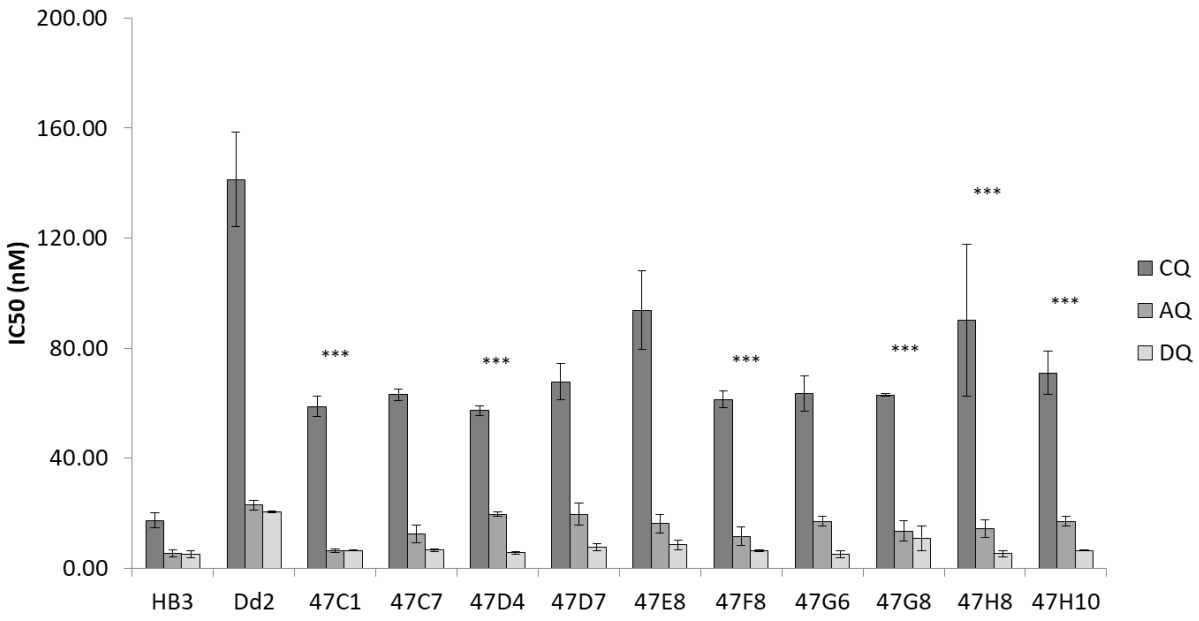

*Figure 3.4 Drug susceptibility of the S47 clonal lines towards CQ, AQ and DQ, including the reference HB3 and Dd2. Ten clones of S47 were chosen from limiting dilution and the IC_{50} values were obtained using the SYBR Green method. The data represent the mean±SEM of at least three to five independent determinations per clones. Two Way ANOVA test was performed to determine significance between isolates and drug at level ***= p<0.001.*

Figure 3.4 shows the drug response of the clonal lines of S47. Ten clones were selected and an *in vitro* assay was performed for all the clones towards CQ, AQ and DQ. Based on the genotyping results by pyrosequencing in Table 3.3, all clones from S47 harboured the CVIET haplotype. However, the IC_{50} values were mostly half of the IC_{50} value of CQ for Dd2. The readings ranged from 57.37 to 93.77 nM for CQ, while AQ ranged from 6.29 to 19.65 nM and DQ from 5.10 to 10.91 nM. All clones were sensitive to DQ but they harboured the *pfcrt* CVIET haplotype. Nevertheless, half of the S47 clones were sensitive to AQ (47C1, 47C7, 47F8, 47G8, 47H8) and another five clones were resistant to AQ (47D4, 47D7, 47E8, 47G6, 47H10). The highest values of IC_{50} for CQ was recorded at 93.77 ± 3.31 nM for 47E8 and the lowest was 57.37 ± 1.77 nM for 47D4. There was no clear cross-resistance within the clones but the closest value was presented by 47E8 when compared to the resistant Dd2 strain (CQ : 93.77 ± 14.26 nM, AQ: 16.26 ± 3.36 nM). The values of the drug response between clones and drugs

showed a significance difference at level p<0.001 when analysed by a Two Way Anova test. Between drugs, CQ and AQ were statistically different and also between CQ and DQ at level p<0.001.

From the above results, only one clone was chosen as a candidate for a further characterization assessment. Interestingly compared to the S9 clones, the S47 clones were genetically resistant but showed intermediate drug susceptibility to CQ when compared to the resistant strain Dd2. Clone 47C7 was selected from this study. This clone showed a phenotype characterized by intermediate resistance to CQ and sensitivity to AQ and DQ.

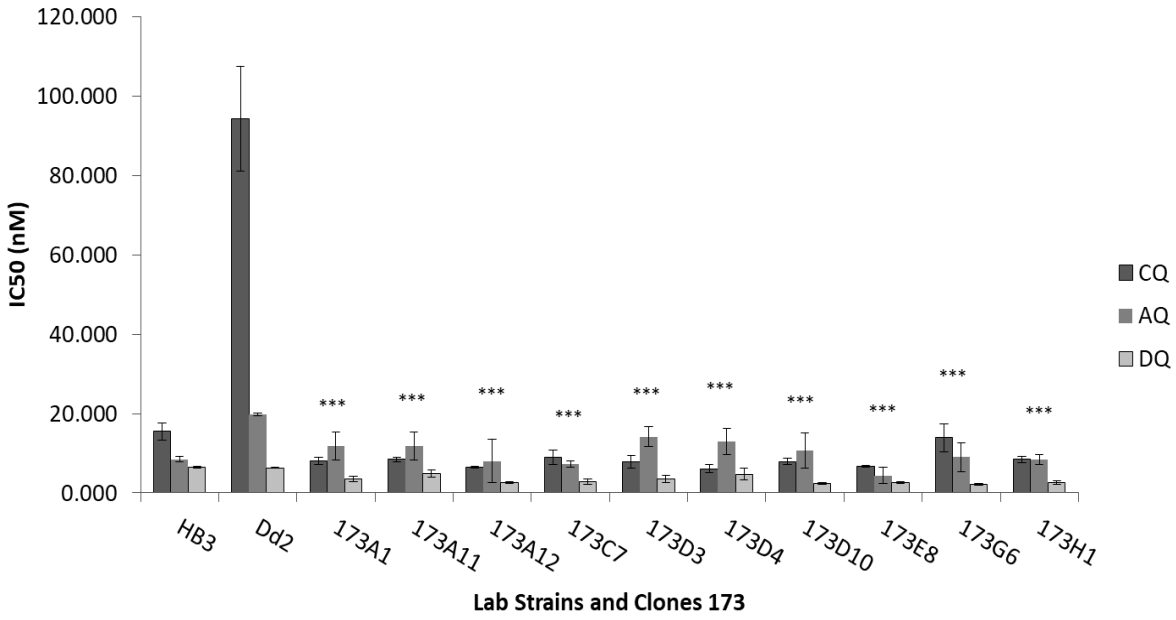

*Figure 3.5 Drug susceptibility of the S173 clonal lines towards CQ, AQ and DQ, including the reference HB3 and Dd2. Ten clones of S173 were chosen from limiting dilution and the IC_{50} values were obtained using the SYBR Green method. The data represent the mean±SEM of five independent determinations per clones. Two Way ANOVA test was performed for significance differences between isolates and drug at level ***= p<0.001.*

Finally, the third set of clones were analysed and these clones were obtained from sample 173 (S173). Ten clones were chosen after a limiting dilution assay and tested in an *in vitro* susceptibility test. The parental clones S173 exhibited a sensitive phenotype towards CQ and DQ but were resistant to AQ. After the analysis was conducted, IC_{50} values of ten clones showed variation between them. There was a significant difference in the drug response value between clones and the drug studied at level $p<0.001$ using the Two Way Anova test. Drug response of all clones were difference statistically compared to Dd2 but not HB3.

The IC_{50} values for CQ ranged from 6.10 to 13.9 nM while those for AQ ranged from 4.34 to 14.20 nM. In contrast, the values for DQ were recorded from 2.20 to 4.88 nM. From Table 3.3, the genotypic analysis has shown that all clones of S173 harboured the *pfcrt* CVMNK haplotype similar to the parental strain. The CVMNK haplotype was associated with CQ-sensitivity. The highest IC_{50} value recorded for CQ was by clone 173G6 at 13.92 ± 3.52 nM and the lowest was 6.10 ± 1.06 nM. The highest value for the AQ drug response was recorded by clone 173D3 at 14.20 ± 2.54 nM. Lastly for the AQ metabolite, DQ, the highest value was shown at 4.88 ± 0.53 nM by clone 173A11.

In this analysis, similarly to the other clone groups, one candidate from the S173 clonal lines will be chosen. From the above results, a selection was made after several consistent results by clone 173D3. The phenotype of 173D3 exhibited CQ-sensitivity and intermediate resistance to AQ. All clones (9C6, 47C7 and 173D3) that were selected from each group were further investigated and analysed by studying the drug accumulation in the food vacuole of the parasites.

3.4.2 Drug accumulation in *P. falciparum*

Phenotype confirmation of the selected clones were further investigated. It involves the amount of drug accumulation, which is defined

by transport and/or partitioning processes in the food vacuole of *P. falciparum.* The parental clones HB3 and Dd2 along with the three selected clones chosen from the drug susceptibility test were incubated in a buffer containing 40 nM [3H]-chloroquine and 40 nM [3H]-amodiaquine to determine the amount of chloroquine accumulation at 5 mins (initial uptake phase) and 20 mins (early steady phase, Fig 3.6 A and C).

The amount of CQ and AQ was expressed as a cellular accumulation ratio (CQ_{in}/CQ_{out}) or (AQ_{in}/AQ_{out}) given as the total amount of CQ taken up in 5 min and 20 min, respectively from 40 nM extracellular concentration, divided by the residual extracellular CQ concentration. The results of these experiments showed that high accumulation of CQ was similar to the parental HB3 whereas low accumulation of CQ was comparable to parental Dd2.

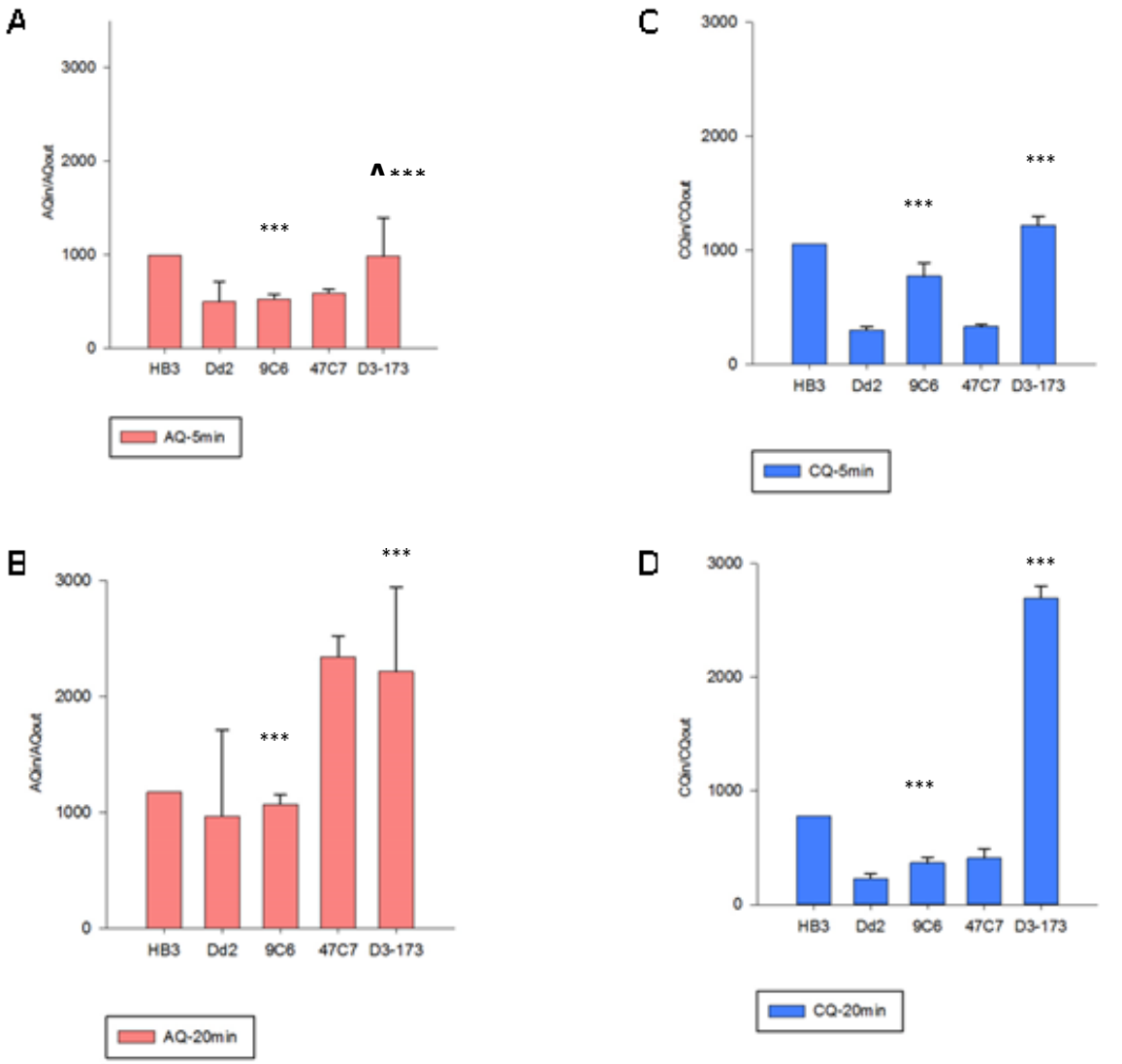

Figure 3.6 CQ and AQ accumulation in reference strain HB3 and DD2 along with the selected clones from the field isolates. ***A*** *and* ***B*** *refer to AQ accumulation at 5 and 20 mins time point in the HB3 and DD2 parental strains together with 9C6, 47C7 and 173D3. While* ***C*** *and* ***D*** *refer to CQ accumulation in the the HB3 and DD2 parental strains together with 9C6, 47C7 and 173D3. The data represents the mean value ± SEM of three independent determinations per clone. A significant difference was observed between the clones at level ***=p<0.001 using Two Way Anova Test for each drug accumulation.*

The results of CQ and AQ for 5 min and 20 min time points are illustrated in Figure 3.6. It appears that clones which accumulated high level of CQ were susceptible to CQ while clones accumulated lower CQ were resistant. It was shown that 9C6 was resistant clone while 173D3 was a susceptible strain. From Two Way Anova analysis, there was a significant

difference (***= p<0.001) between the clones tested. However in this experiment the parental strain (positive control), has a high variation for Dd2 at AQ 20 mins therefore more replicates were required to confirm the phenotype of the clones. Sa et al., (2009) and Sanchez et al., (2011) reported similar findings where based on the experimental results, susceptible clones accumulated higher amounts of AQ while the resistant clones accumulated lower amount of AQ Figure 3.6 (A and B). The driving force for parasite accumulation of the 4-aminoquinolines is proposed to be a transmembrane proton gradient sustained by a vacuolar ATPase (Hawley et al., 1999).

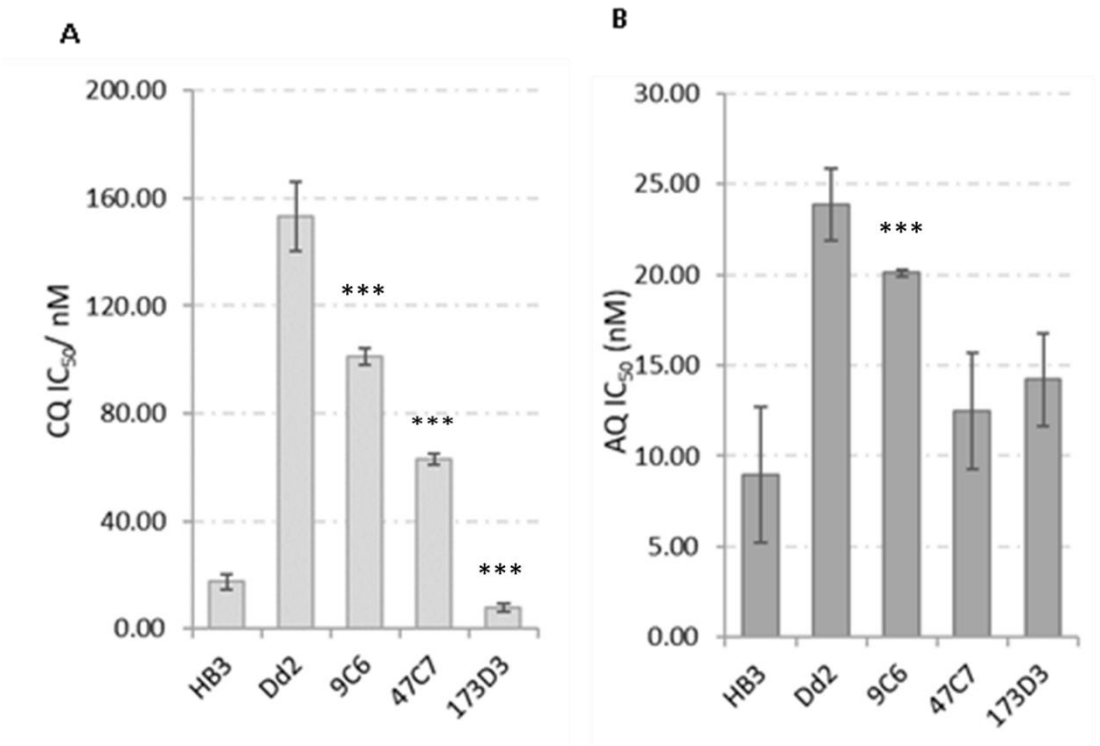

*Figure 3.7 Drug susceptibility of parental clones HB3 and Dd2 along with the selected clones. A and B refers to CQ IC_{50} and AQ IC_{50} respectively. The data represents the mean value ± SEM of three independent determinations per clone. A significant difference was observed between the clones at level ***=p<0.001 using One Way Anova Test for CQ drug susceptibility assay while for AQ only 9C6 was statistically different compared to other clones.*

Another observation reported by Sanchez et al., (2011) was that the AQ accumulation ratios were much higher than CQ in the progeny studied. Similar observations were obtained in this study but more replicates are needed to validate and to reduce the variations. In another study by Hawley et al., (1999), AQ accumulated 2-3 fold higher compared to CQ. These authors also described high AQ IC_{50} values that were associated with lower amounts of the drug and the two response parameters reciprocally correlated to each other. This supports the findings in this study where the drug susceptibility of CQ and AQ reciprocally correlated which means higher IC_{50} values with lower amount of drug accumulation (Figure 3.7)

Further investigation was performed to confirm the phenotype of 9C6, 47C7 and 173D3. We hypothesized that 9C6, 47C7 and 173D3 were adapted to the culture condition since it was cultured continuously for a longer period in the laboratory. New set of clones from 9C6, 47C7 and 173D3 freeze downs, dated 23.12.12, 23.12.12 and 14.04.12 respectively, were obtained, thawed and cultured. These clones were labelled as 9C6O, 47C7O, 173D3O where O referred to 'recent'. After two cycles, the trophozoites of the older clones were used for the drug transport assay. Similarly, in parallel the adapted clones were tested as described in chapter 2.0 Section 2.7.7.

The results are shown in Figure 3.8. From the AQ drug accumulation assay (A and B), it was observed that 9C6 and 9C6O were resistant compared to the parental strains (positive control). The drug accumulation in the 9C6 and 9C6O were lower compared to 47C7, 47C7O, 173D3 and 173D3O. However, variations were high in each clones, therefore more determinations for each clones were needed. These results were not consistent with CQ accumulation assay. In the graphs (C and D), 9C6 was not resistant for early and late phase while 9C6O were resistant when compared to the parental clone, Dd2. On the other hand, 47C7 and 47C7O had lower drug accumulation ratio and they were resistant compared to Dd2 while 173D3 and 173D3O, they were susceptible clones since the drug

accumulation ratio were much higher compared to other clones. However, statistically there were no significant difference between the adapted and recent clones. Variations in some clones of the drug accumulation assay were high and this made the phenotype confirmation difficult.

Further analysis and study should be performed to validate the phenotype of the isolated clones from Burkina Faso in order to understand the resistance mechanism of AQ resistance

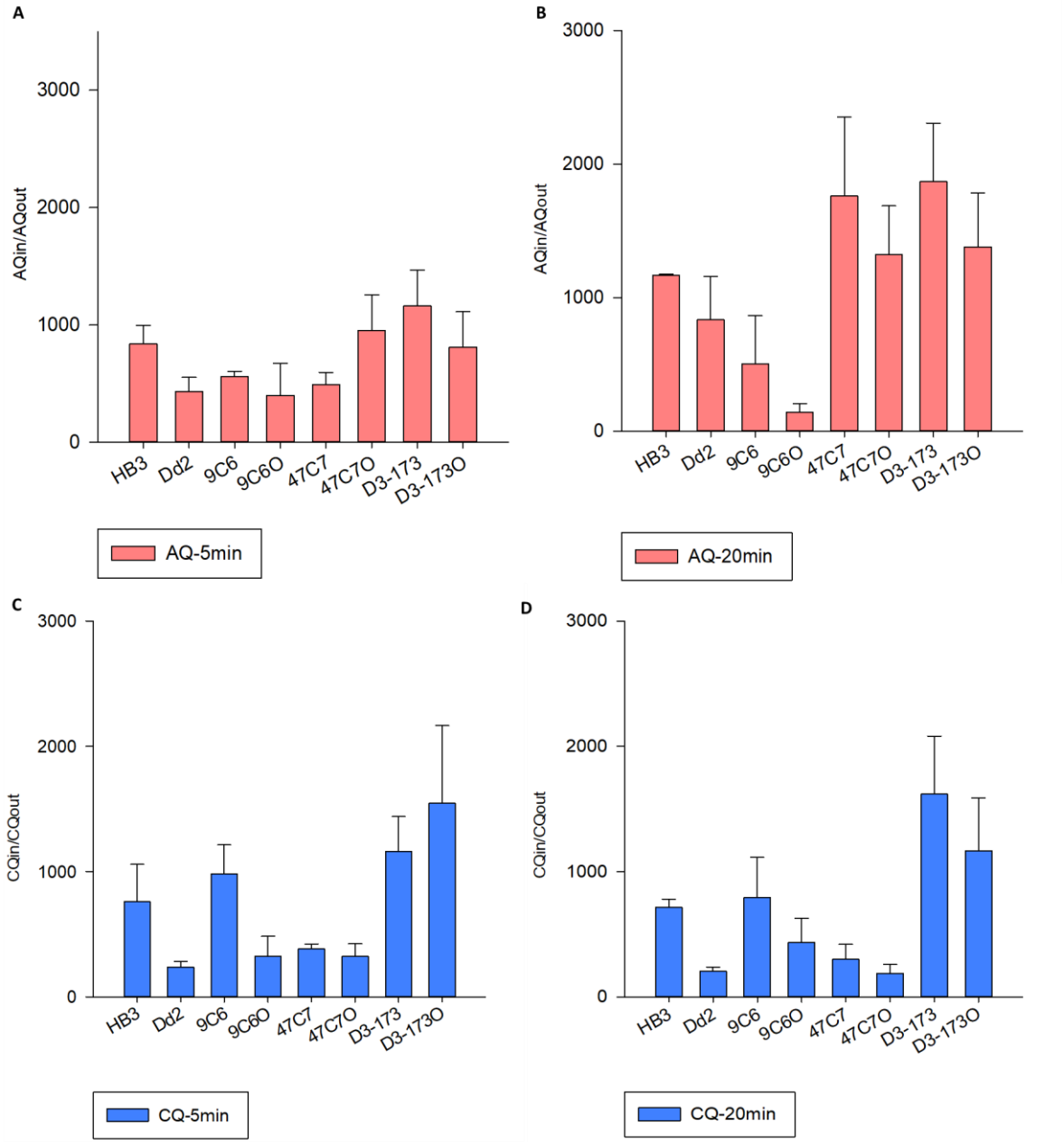

Figure 3.8 CQ and AQ accumulation in reference strain HB3 and DD2, along with adapted clones, 9C6, 47C7, 173D3 and recent clones, 9C6O, 47C7O, 173D3O. ***A*** *and* ***B*** *refer to AQ accumulation at 5 and 20 mins time point in the HB3 and DD2 parental strains together with adapted and recent clones. While* ***C*** *and* ***D*** *refer to CQ accumulation in the HB3 and DD2 parental strains together with adapted and recent clones. The data represents the mean value ± SEM of at least three to five independent determinations per clone. Significant difference was observed between the adapted and recent clones at level *** = p<0.001 using Two Way Anova Test for each drug accumulation.*

3.4.3 Correlations between drugs

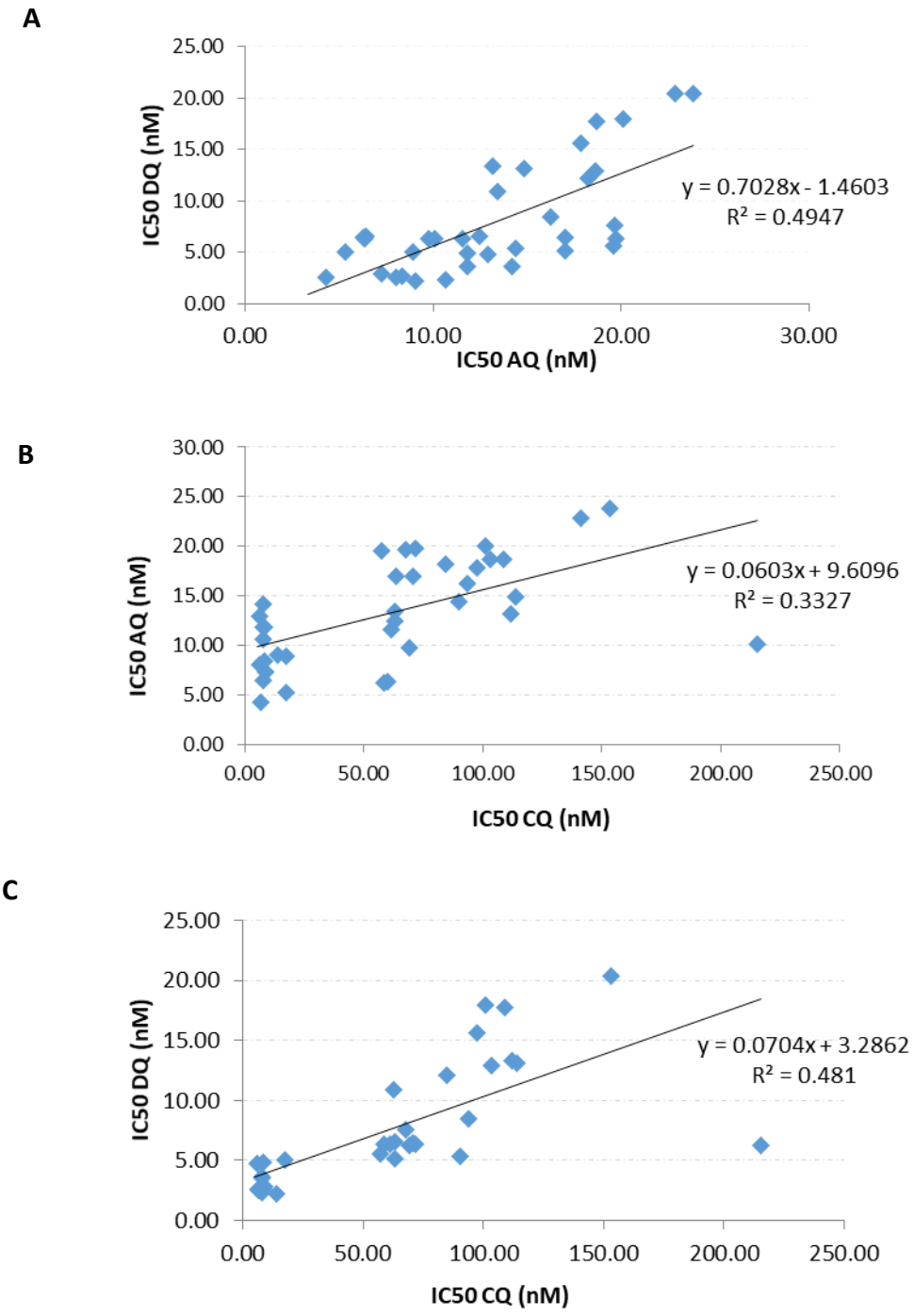

Figure 3.9 Correlation between drugs based on the IC_{50} values. The IC_{50} values of all clones were tabulated in the graphs to understand the relationship between the drugs. A. Desethyl-amodiaquine vs Amodiaquine B. Amodiaquine vs Chloroquine C. Desethyl-amodiaquine vs Chloroquine

A moderate correlation between AQ and DQ (R^2=0.4947) was observed since DQ is the main metabolite of AQ and is responsible for the main antimalarial activity of the drug. DQ has a longer half time and higher concentration-time profile compared to the parent compound (Laurent et al., 1993). A weak positive correlation was observed between AQ and CQ (R^2=0.3327) in this study, however more data is needed to confirm the association between these drugs. Although cross-resistance exists, it was not so high in the clonal lines. A significant correlation between CQ and AQ as well as DQ is plausible, since resistance to CQ has compromised the efficacy of AQ in the field. Nonetheless, positive correlation does not automatically provide any evidence for cross-resistance as previously observed for 4-quinolinemethanols mefloquine and quinine (Elueze et al., 1996). A few *in vitro* studies have reported a positive correlation between AQ and CQ. Besides that, cross-resistance between DQ and CQ has been observed in *in vitro P. falciparum* isolates from various regions of Africa (Basco et al., 2003; Ringwald et al., 1998), South America (Childs et al., 1989; Watt et al., 1987) and Southeast Asia (Segurado et al., 1987).

3.4.4 Clustering of clonal parasites based on IC_{50}

A dendrogram is a way to visually describe the relationships between samples, genes or isolates. The word dendogram originated from a Greek word *dendro* means "tree" and *gramma* means "drawing". The tree diagram is frequently used to illustrate the arrangement of the clusters produced by hierarchical clustering (Everitt, 1998).

A cluster analysis was established based on the IC_{50} values of CQ of the clonal lines and classified all isolates into two clusters, A and B. The two groups consisted of clonal lines from S173, which were identified previously as sensitive clones, and clonal lines of resistance strains from S47 and S9, with different level of distribution.

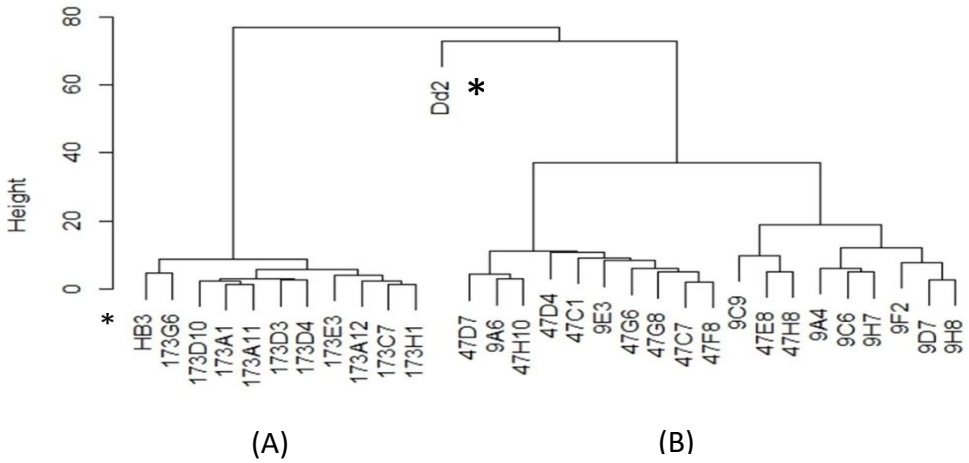

Figure 3.10 Dendogram of clonal lines from parental clones S9, S47 and S173 produced two main cluster, cluster A and B which refers to a sensitive group and resistance group respectively.

All clones from S173 were grouped and closely related to HB3 thus indicaing that all clones were sensitive to CQ. From the above cluster, most clones of S47 and S9 were closely related; however cluster B showed dissimilarities to cluster A. This analysis supported the drug response assay and distinguished sensitive or wild-type and resistant strains.

3.5 Dormancy

The first approach used in finding AQ resistant parasites described in the previous section resulted in the isolation of three clones with different phenotypes: 9C6, 47C7 and 173D3. Well-characterized isolates are vital to study the mechanism of drug resistance. Therefore, more candidate clones were needed to achieve the aim of this study.

Thus, we performed another strategy whereby we challenged the field isolates with a certain amount of desethyl-amodiaquine (DQ). The cut-

off value for DQ based on Basco et al., (2002) and WHO (2007) was >60 nM. A total of 200 samples from Nouna which were positive by microscopy within the study period were selected. The parasites were cultured as described in section 2.6.1. Unfortunately, no parasites survived the drug concentration used and the cultures were maintained for a maximum two months before being discarded.

Surprisingly, during the *in vitro* resistance study, after the ring-stage parasites were treated with DQ drugs, they developed a round morphology, and retained blue cytoplasm and red chromatin, as observed by a thin Giemsa smear. We suggested that the parasites with this morphology were dormant parasites. Tucker et al., (2012), reported this finding with similar morphologies of parasites that were observed by a thin Giemsa smear as shown in Figure 3.11 after exposure to artemisinin.

A preliminary study was performed to confirm the dormancy of these parasites using quantitative recrudescence assays. The procedures were described in section 2.7.8. Six samples suspected to habour dormant parasites shared similar observations as shown in Figure 3.11.

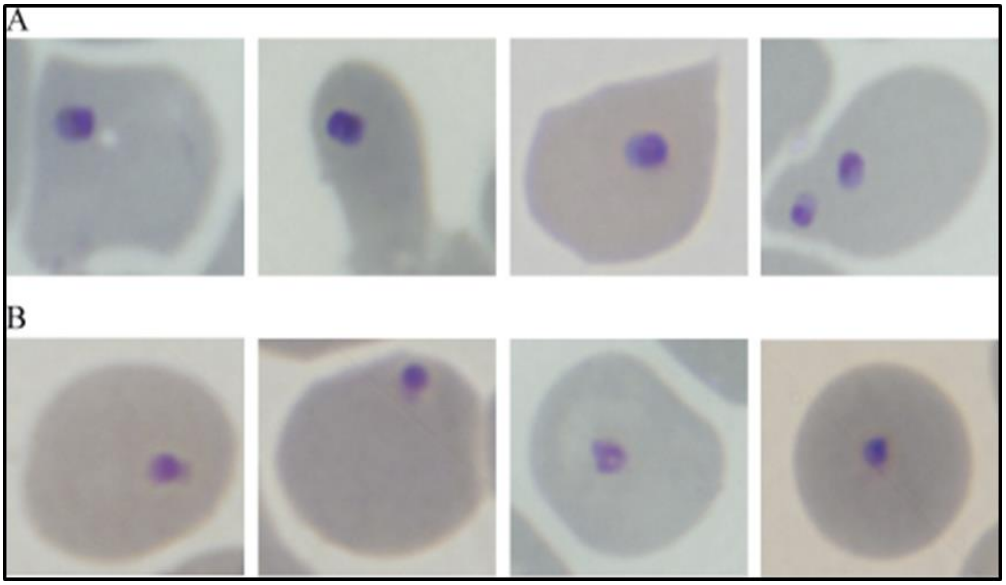

Figure 3.11 Comparison of dormant and dead parasites after a typical exposure to artemisinin drugs. (A) After ring-stage parasites were treated with artemisinin drugs, they became dormant, developed a rounded morphology, and retained blue cytoplasm and red chromatin on a Giemsa smear. (B) Dead or crushed parasites do not have distinct chromatin and cytoplasm, appearing globular, and they appeared red/purple on a Giemsa smear (Tucker et al., 2012)

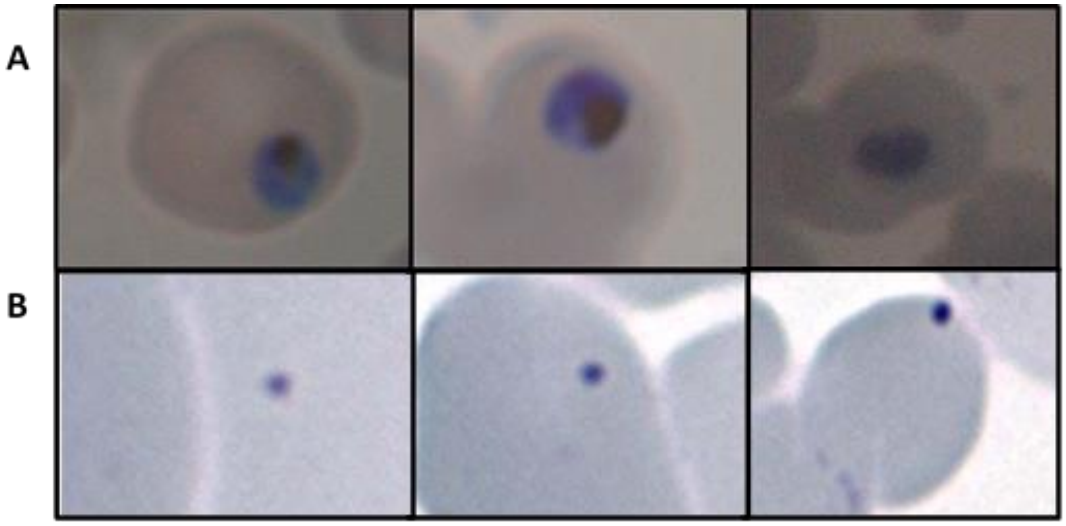

Figure 3.12 Comparison of suspected dormant and dead parasites after exposure of DQ drug in this experiment for culture labelled R12-234 (A) After ring-stage parasites were treated with DQ drug, they were suspected to become dormant, formed a rounded shape with blue cytoplasm retained and red chromatin on a Giemsa smear. (B) Globular shaped dead parasites observed without distinct cytoplasm and chromatin.

As displayed in Figure 3.12, the suspected dormant parasites (A) showed different morphologies compared to dead parasites (B). These features or morphologies remained unchanged even after the drug was taken off from the culture. These parasites were maintained in cultures for a maximum two months; however no AQ resistant parasites were recovered from after drug challenge. The suspected dormant parasites were assumed dead after being challenged with 60 nM DQ and they looked like dormant parasites that were challenged by artemisinin.

In conclusion, the summary of the findings of this study and characteristics of the selected clones that were recorded and hypothesized are listed below.

Table 3.4 Summary of the characteristics of selected clones from Nouna Burkina Faso based on the genotypic and phenotypic analysis.

Test		Selected clones		
	Drug	**9C6**	**47C7**	**173D3**
Genotyping	-	Resistance	Resistance	Sensitive
Drug response	CQ	Resistance	Intermediate Resistance	Sensitive
	AQ	Resistance	Sensitive	Intermediate Resistance
	DQ	Resistance	Sensitive	Sensitive
Drug Accumulation (Figure 3.6 & Figure 3.8)	CQ	Resistance	Resistance	Sensitive
	AQ	Sensitive	Sensitive	Sensitive
	CQ	Sensitive	Resistance	Sensitive
	AQ	Resistance	Sensitive	Sensitive
		9C6O	**47C7O**	**173D3O**
	CQ	Sensitive	Resistance	Sensitive
	AQ	Resistance	Sensitive	Sensitive

4.0 Discussions

Bourasso village is situated within the area of the Nouna Health and Demographic Health Surveillance System (HDSS), which includes nearly 80,000 inhabitants under continuous demographic surveillance. This has been used as a sample frame for many epidemiological and clinical studies (Kouyate et al., 2008; Louis et al., 2012). In this study, the sampled data showed a decreased parasite prevalence during six sampling period from 2000, and between 2009 and 2012. This observation supports previous findings in which reduced malaria incidence was also described in East African regions such as Tanzania and Kenya (Schellenberg et al., 2004; O'Meara et al., 2008; Mmbando et al., 2010). Malaria incidence in Burkina Faso was reported by WHO (2011) as still rising and Beiersmann et al., (2011) also reported a reduction of parasite prevalence in children less than four years old between the rainy and dry seasons from 1999 to 2009 after the increased use of insecticide-treated bed nets (ITNs) in the population.

In parallel to this study, Geiger et al., (2012) reported that asymptomatic malaria was more common during the dry season (April 2010-2012) perhaps due to a reduced abundance of *P. falciparum*. The features used as identification for asymptomatic malaria were axillary body temperature of < 37.5˚C at presentation with no history of fever and *Plasmodium* infection confirmed by positive microscopy. In contrast, symptomatic malaria was more frequent during the rainy season with a high parasite density (>1000 ul/parasites) and it was observed that single and mixed infection with *P. malariae* declined significantly during the study period. A similar trend was reported in Malawi, where higher *P. falciparum* was link to higher prevalence of minority species (Bruce, 2008) and another study in Burkina Faso showed that the implementation of ITNs has reduced the prevalence of *P.malariae* significantly more than *P. falciparum* (Hableutzel et al., 1999).

Parasite prevalence of *P. malariae* during the dry season in April was very low (<1000 parasites/ul) and a similar observation was reported in another study conducted 230km in the south of Burkina Faso, near Bobo-Dioulasso, where *P. malariae* infection was reduced by half during the dry season compared to the rainy season (Boudin et al., 1999). *P. ovale* infection was very uncommon and only

restricted to high-transmission of malaria during the monsoon season. *P. malariae* may not be as terminal as other *Plasmodium* species but it can cause chronic infection by remain in the human host at a low level for an extended period of time (Mohapatra et al. 2008; Collin et al., 2007). This could possibly lead to an underestimation of the parasite burden, as the detection limit is low for microscopy. The use of molecular tools such as PCR in this study was more more sensitive in detecting low levels of other *Plasmodium* species, such as *P. malariae* and *P. ovale.*

Serum samples from patients were analysed for AQ and DQ concentrations. Liquid chromatography has been known to have precision and accuracy in determining the major metabolites of AQ and DQ when administered to patients with uncomplicated malaria (Adedeji et al. 2015). Determination of AQ and DQ concentration in the serum was important to determine the resistant parasites. The methodology was established by the Department of Pharmacology/Epidemiology, University Hospital of Heidelberg. Optimization was accomplished by monitoring varying chromatographic conditions in terms of chromatographic columns, mobile phase systems, extraction solvents and solvent for reconstitution before producing an appropriate condition that gave satisfactory results. It is crucial to use the correct mobile phase used during analysis to obtain good resolution between the peaks and to produce analytes with clear sharp peaks, without interference from endogenous substances (Adedeji et al. 2015). The mobile phase was capable of separating the IS and analytes within 6.0 min, with the UV absorption spectrum set at 340nm. 2(7-Isoquinolinyl)-Ethanol was selected as the IS because it did not interfere with the analytes. The retention times of the drugs on the chromatogram were 4.5 min for IS, 5.7 min for DQ and 7.6 min for AQ.

Cultivation of the field isolates was a tedious and laborious task (Basco, 2007). Field isolates could appear as early as two days after thawing or as late as 4 weeks. Some field isolates took longer to adapt to the culture condition and reproduce. Field isolate cultures , which were negative for parasite, after 8 weeks were discarded.

Pyrosequencing is a reliable technique to determine short DNA sequences. However, errors can also occur. In a parallel study by C. Geiger in our laboratory, her

data showed parental clones S256, sequenced from cDNA was a sensitive strain. This supports the drug susceptibility assay in Figure 3.2, which has the value of 31.99 ± 3.71nM against CQ and 9.74±1.49nM against AQ. However, the genotypic analysis using pyrosequencing showed, that S256 harbours a CVIET pfcrt haplotype, which is a hallmark of a resistant strain (Table 3.2).

Variation in IC_{50} values or in vitro assays has been reported (Nzila, 2010) due to interlaboratory (interassay) variation when producing either IC_{50} or IC_{90}. Several aspects or conditions could affect the test during parasite cultivation, such as initial parasitemia, haematocrit, incubation time, serum and gas mixture composition. A right shift in the drug response curve can only be accepted if all the in vitro variables remain constant and no interassay variation is present. A universally accepted or standard protocol for the in vitro sensitivity assay needs to be established to allow comparison of results between different laboratories possible

Table 4.0 Thresholds used to define in vitro resistance of *Plasmodium falciparum* to amodiaquine

Threshold	Method	Reference
IC_{50} ≥ 4 nM	Isotopic	Chaparro & Wasserman 1999
IC_{50} ≥ 30 nM	Schizont maturation	Childs et al. 1989
IC_{50} ≥ 59 nM	Isotopic	Basco et al. 2002
IC_{50} ≥ 60 nM	Isotopic	Reynes et al. 1997
IC_{50} ≥ 80 nM	Isotopic	Pradines et al. 1998, Rason et al., 2002
	Schizont maturation	Ringwald et al. 1996
IC_{50} ≥ 400 nM	Schizont maturation	Segurado et al. 1997
MIC ≥ 4 nM of blood	Schizont maturation	Draper et al. 1988
IC_{90} ≥ 400 nM of blood in non immune-populations	Schizont maturation	Wernsdorfer & Paine 1988
IC_{99} ≥ 400 nM of blood in immune population	Schizont maturation	Wernsdorfer & Paine 1988

Three different phenotypes of *P. falciparum* (S9, S47, S173) based on the IC_{50} values were cultured and the clonal lines were obtained. All clones from S9 and S47 harboured *pfcrt* CVIET haplotypes, while all clones from S173 were CVMNK haplotype.

The clones from S9 showed higher IC_{50} values on average against CQ and AQ compared to clonal parasites of S47. While some clones were sensitive to DQ for S9 and all were sensitive in clonal lines of sample 47. Clonal parasites from S173 had lower IC_{50} values against CQ. Responses to CQ, AQ and DQ varied between the clones, perhaps due to the difference in genetic background.

Clear cross-resistance between CQ and AQ was observed in clonal lines S9 eg 9C9, 9C7, 9H8 and in clone S47E8. Cross-resistance was detected, however it was not high in the clonal lines. CQ and AQ are structurally quite similar, with the main difference, being that AQ has a phenolic substitution in the side chain, we expect some degree of cross-resistance. Nevertheless, studies of cross-resistance between CQ and AQ *in vitro* have been inadequate; a few studies have revealed cross-resistance between the two drugs (Scott et al., Geary et al., 1987), but others have not shown any cross-resistance (Geary et al., 1983). In vitro analysis of cross-resistance between drugs is essential to prevent the development of introduction of new drugs that are already resistant in the field (Fall et al., 2016).

Antimalarial drug susceptibility and resistance to QN, AQ, piperaquine, and lumefantrine are influence by variation in the PfCRT protein. CQ shows cross-resistance with AQ and QN, which is primarily mediated by K76T mutation. Mutations at 72–76 codons at PfCRT confer higher resistance against CQ and medium level of resistance against AQ in Southeast Asia and Africa. However, these mutations are associated with greater AQ resistance in South America. Therefore, not only is the K76T mutation in the PfCRT protein is an effective molecular marker for the antimalarial drug, their previous usage in the region should also be taken into account (Sa et al., 2009).

AQ resistance was observed in S9 clones where the IC_{50} values were lower than the parental clone. Nevertheless, consistent results of the phenotypic analysis need to be obtained for validation. Adaptation to laboratory conditions can occur in resistant parasite strains and they can lose their resistant phenotype (Nzila et al., 2010). Long term and continuous culture is not recommended for drug testing due to the instability of the

resistance phenotype. Unstable phenotypes may be associated with reduced parasite fitness, meaning once drug pressure was removed, the phenotype reverts back to normal (Nzila et al., 2010). Another study by Basco (2006) reported adaptation to goat serum for long term cultivation, and different batches of goat sera were responsible for the conflict between the obtained results. Nevertheless, more independent determinations are required for accurate results and to validate these observations.

AQ resistance was intensely observed in certain regions (Mandi et al., 2008), including North-Western Burkina Faso (Danquah et al., 2010) which then may endanger the efficacy of AQ-artesunate treatment regime. As mentioned previously, DQ, the main metabolite of AQ, plays a major role in the antimalarial activity of the drug. This is due to the higher concentration-time profile and the longer half-life of DQ compared to those of the parent compound, AQ (Laurent et al., 1993).

An increase in DQ resistance, measured using vitro susceptibly assays was also discovered in Dakar, Senegal where the number of resistant cases rose from 5.6% in 2013 to 30.6% in 2014, (Fall et al., 2014). To date, the AQ-AS is the world's second most widely used artemisinin-based combination therapy (ACT) (Zwang et al., 2009).

Many previous studies reported on the genetic linkage between CQ resistance and *pfcrt* in the HB3 ¥ Dd2 cross (Wellems *et al*., 1991; Fidock *et al*., 2000; Sa *et al*., 2009; Patel *et al*., 2010). Sanchez et al., (2011) stated that the progeny, which accumulated higher drug levels - showed lower IC_{50} values, but the progeny that accumulated less chloroquine tend to, but does not necessarily always have higher IC_{50} values. Previous studies on CQ resistance suggested a reciprocal correlation between susceptibility and drug accumulation in *P. falciparum* (Fitch, 1970; 1973). Polymorphisms within *pfmdr1* did not significantly affect susceptibility to or accumulation of AQ. PfMDR1 mutations at the N86Y and N1042D positions have been shown to be associated with AQ resistance (Sa et al., 2009). K76T and A220S mutations in the *pfcrt* gene and a N86Y mutation in the *pfmdr1* gene are associated with high resistance to CQ in field isolates.

pfcrt and *pfmdr1* are mutated genes in *P. falciparum* that respond to selection by quinoline antimalarial drugs (Fidock et al., 2000; Sa et al., 2009; Sanchez et al., 2010; Okombo et al., 2011). These two genes encode proteins of the parasite's digestive vacuolar membrane. The mutations are believed to confer resistance to quinolines by inhibiting these drugs from accumulating in the digestive vacuole to the concentrations required to inhibit endogenous haem detoxification processes (Fitch, 2004).

PfCRT and PfMDR1, are most likely to assist the drug movement in opposite directions (Sanchez *et al*., 2010). When mutated PfCRT functions either as a carrier (Sanchez *et al*., 2004; Summers and Martin, 2010) or as a voltage gated channel of drugs (Bray *et al*., 1996; Paguio *et al*., 2009), facilitating the efflux of these drugs from the digestive vacuole. On the other hand, PfMDR1 seems to act as a drug importer, substitution of polymorphic amino acids reduces the ability of PfMDR1 to transport a given drug into and therefore concentrates it in the digestive vacuole (Rohrbach *et al*., 2006; Sanchez *et al*., 2008a).

Interaction between PfCRT and PfMDR1 in a resistant phenotype is not fully understood. Several different *pfcrt* and *pfmdr1* alleles have emerged in *P. falciparum* strains in different geographic settings, which makes it complicated to understand their relationship. These different polymorphic forms are caused by independent founding events and seems to reflect distinct solutions to regional histories of drug use and hence drug selection (Sa *et al*., 2009; Wellems *et al*., 2009). *pfcrt* and *pfmdr1* alleles from South America have arisen in an area where AQ use has preceded that of chloroquine (Sa *et al*., 2009).

In this study, there was a moderate correlation between AQ and DQ (r^2=0.495) and a weaker correlation between AQ and CQ (r^2= 0.333). Bray et al., (1996) found that the susceptibility or AQ strains was correlated to the susceptibility of CQ (r^2= 0.96) and there was a correlation between the accumulation of AQ with accumulation of CQ (r^2= 0.94). Accumulation of both AQ and CQ resistance was significantly reduced in CQ resistant isolates. From the results of the genotyping and drug response tests that were performed in this study, we concluded that 9C6 was resistant to CQ, AQ and DQ,

47C7 showed an intermediate resistant to CQ, sensitive to AQ and DQ while 173D3 was sensitive to CQ and DQ, and intermediate resistance to AQ. Additionally, in the drug accumulation test, the results were not consistent with the drug response test especially for 9C6/9C6O, while 47C7/47C7O were resistant to CQ and sensitive to AQ whereas 173D3 was sensitive to both CQ and AQ.

Selection of AQ-resistant parasites was unsuccessful in the *in vitro* selection strategy challenged with 60 nM DQ. One of the reason could be that there was no resistant strains in the samples collected, or the number of AQ-parasites in the population was still very low. Another reason might be that the drug concentration used during the challenge was too high. 60 nM of DQ was the cut-off value used as suggested by Basco et al., (2002) and WHO (2007). The method used to determine IC_{50} in our laboratory was by Sybr Green1 while Basco et al., (2002) was using an isotopic method (Table 4.0). This interlaboratory variation could contribute to the discrepancy in cut off value to determine AQ resistance (Basco et al., 2004; Briolant et al., 2007). An alternative approach to select for AQ resistant parasites is by culturing susceptible parasites *in vitro* with continuous exposure to increasing sublethal drug concentrations (Lambros et al., 1984; Odoula et al., 1988).

In this study, while performing *in vitro* selection using DQ, the parasites exhibited a morphology similar to ART dormant parasites. They were assumed to enter the dormancy state after treatment with DQ. After further investigation, there was no recrudescence observed after the drug was removed. Dormant parasites were reported to be frequently associated with artemisinin-derivates (ART) monotherapy treatment (Meshnick et al., 1996). One plausible reason for the failure of ART monotherapy was related to the ART-induced dormancy phenomenon (Teuscher et al., 2010; Witkowski et al., 2010, Codd et al., 2011), in which ART-treated ring-stage parasites undergo growth arrest and manage to survive and appear again when the drug was removed or decreased (LaCrue et al., 2011; Tucker et al, 2012). In a dormancy state, the metabolic activities were still maintained in the apicoplast and mitochondria, despite a general down-regulation in transcription of gene encoding important enzymes in numerous metabolic pathways (Chen et al., 2014; Peatey et al., 2015). The mechanism of ART-induced dormancy is not yet understood but seems to

be similar to cell cycle arrest in a mammalian cell. The cell cycle regulators such as CDKs and cyclins could play a major role in parasites entering and leaving dormancy.

Gray et al., (1996) hypothesize that after the exposure to DHA, there was an up-regulation of *PfCRK1* and *PfCRK4* to arrest the development of the cells at G1 phase. Parasites also decrease the transcription and expression of *PfMRK* genes and other CDKs to prevent the initiation of DNA replication and, later, the parasites fail to make a transition from G1 to S phase. Parasites arrested at the G1 phase and stayed dormant for a few weeks until the pressure is removed. The data obtained suggests a new antimalarial drug target and also a molecular marker for dormant parasites. This is a significant finding in the understanding of the mechanism of DHA-induced dormancy and open new prospects to avoid recrudescence following artemisinin treatment.

5.0 Outlook

To date, malaria parasites have developed resistance towards most antimalarial drugs. This includes artemisinin derivates in Cambodia (White, 2008), which poses a threat to artemisinin-based combination therapy (ACT). Selecting and tracking resistant parasites from field isolates is difficult, challenging and time consuming, although it can provide critical and useful information on the genetic basis of drug resistance. Such information can help to predict, anticipate and control the spread of resistance. For example, discovery of the gene *pfmdr1* which confers mefloquine resistance resulted from *in vitro* studies (Nzila, 2010).

To study the mechanisms of resistance, we need to find well-characterized drug-resistant strains. However, such strains are not accessible for most antimalarial drugs. Rodent malaria models such as *Plasmodium chabaudi, Plasmodium yeolii, Plasmodium berghei* and *Plasmodium vinkei* have been used extensively as alternatives to study drug resistance in vivo and aid selection of drug-resistant parasite lines for drug mechanisms research (Peters, 1987). Murine models can have limitations, since mechanisms of CQ and artemisinin resistance may differ compared with *P. falciparum* (Hunt 2007).

Several strategies and methods could be improved for future studies to select for AQ-resistant parasites. *In vitro* selection strategies are another potential approach but the time taken to produce the resistant strain is very long and slow. The risk of contamination and slow parasites growth limits the effort in selecting resistant parasites. Increasing the concentration of DQ in *in vitro* selection strategy might introduce additional mutations with time and the underlying mechanism of AQ resistance could be resolved. Thus, in the absence of a well-characterized drug-resistant strains, the mechanism of AQ resistance still awaits to be characterized.

In this study, three different clones were characterized using two response parameters and genotyping as discussed in the previous chapters. Further investigations to validate the phenotype are required. Many classes of compounds have been identified to reverse CQ resistance or re-sensitize to CQ sensitive levels such as verapamil. This is a CQ chemo sensitizer and a blocker for PfCRT (Henry et al., 2006,

Sanchez et al., 2004). Reversibility testing using verapamil could be used to confirm the isolated resistant strains.

Tracking or selecting AQ-resistant parasites could be achieved by several means, such as by increasing the sample size to increase the probability of finding the phenotype of interest. As mentioned above, the task is very laborious and time consuming, hampered by the slow growth of parasites and potential risk of culture contamination. Highly trained personnel, well-equipped laboratory facilities and financial investment would be required. Previous studies have reported, understanding the relationship between *pfcrt* and *pfmdr1* regarding resistance is partly understood. Several different *pfcrt* and *pfmdr1* alleles have emerged in *P. falciparum* strains in different geographic settings (Sa et al., 2009; Wellems et al., 2009). Using this evidence, the samples used for tracking AQ-resistant parasites should come from several different regions with different histories of drug use, such as South America, Asia and Oceania.

Both drug response parameters, *in vitro* susceptibility and drug uptake are vital tools to measure parasite response to drugs. Replicates or several independent determinations of each experiments are essential to obtain accurate results and reduce variations. In this study, it is recommended to perform five to six independent determinations for each experiment. Unstable resistant clones can lose their phenotype if continuously cultivated for a very long period and become culture-adapted parasites. To avoid losing resistant parasites, using freshly cultured clones and performing the assays after two cycles is recommended.

Different laboratories used different protocols for in vitro drug sensitivity assays and a universally-accepted protocol is not available. The need for a standardized assay to allow data comparison between laboratories should be considered. To overcome this situation, a researcher should choose one protocol that best satisfies his or her needs. After the choice is made, it is necessary to establish and maintain the protocol over time so that important findings can be deduced such as trends and the emergence of drug resistant isolates (Basco, 2007).

Conventionally, classical linkage mapping has been using genetic crosses to examine the patterns of drug resistance phenotypes. Later technology, such as whole genome sequencing (WGS) has provided a powerful alternative to study drug resistance and may generate a few numbers of candidate gene regions involved in drug resistance. If such a study obtained clear and well-characterized AQ-resistant clone, WGS would be another choice to determine genes responsible for the AQ resistance

6.0 References

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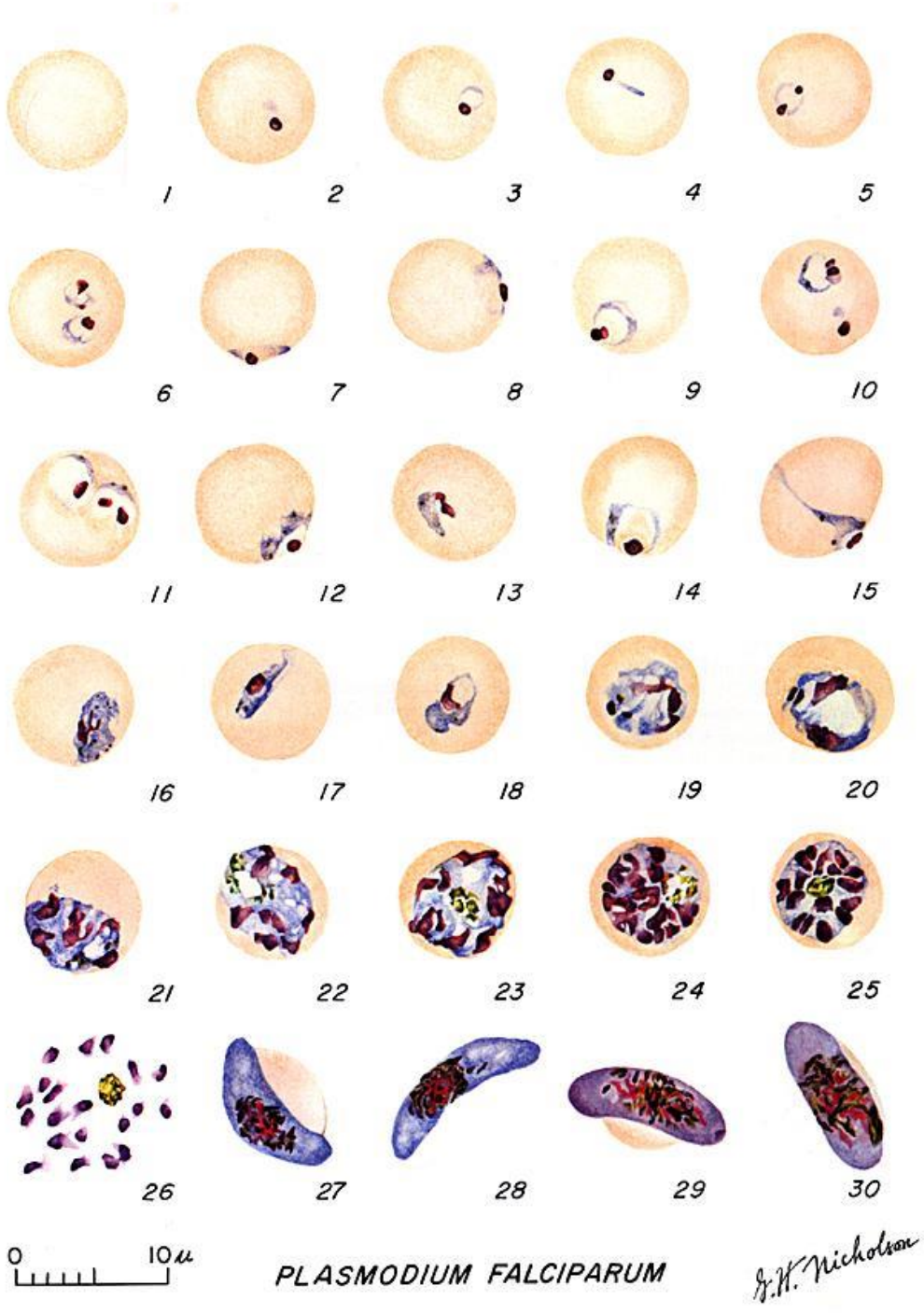
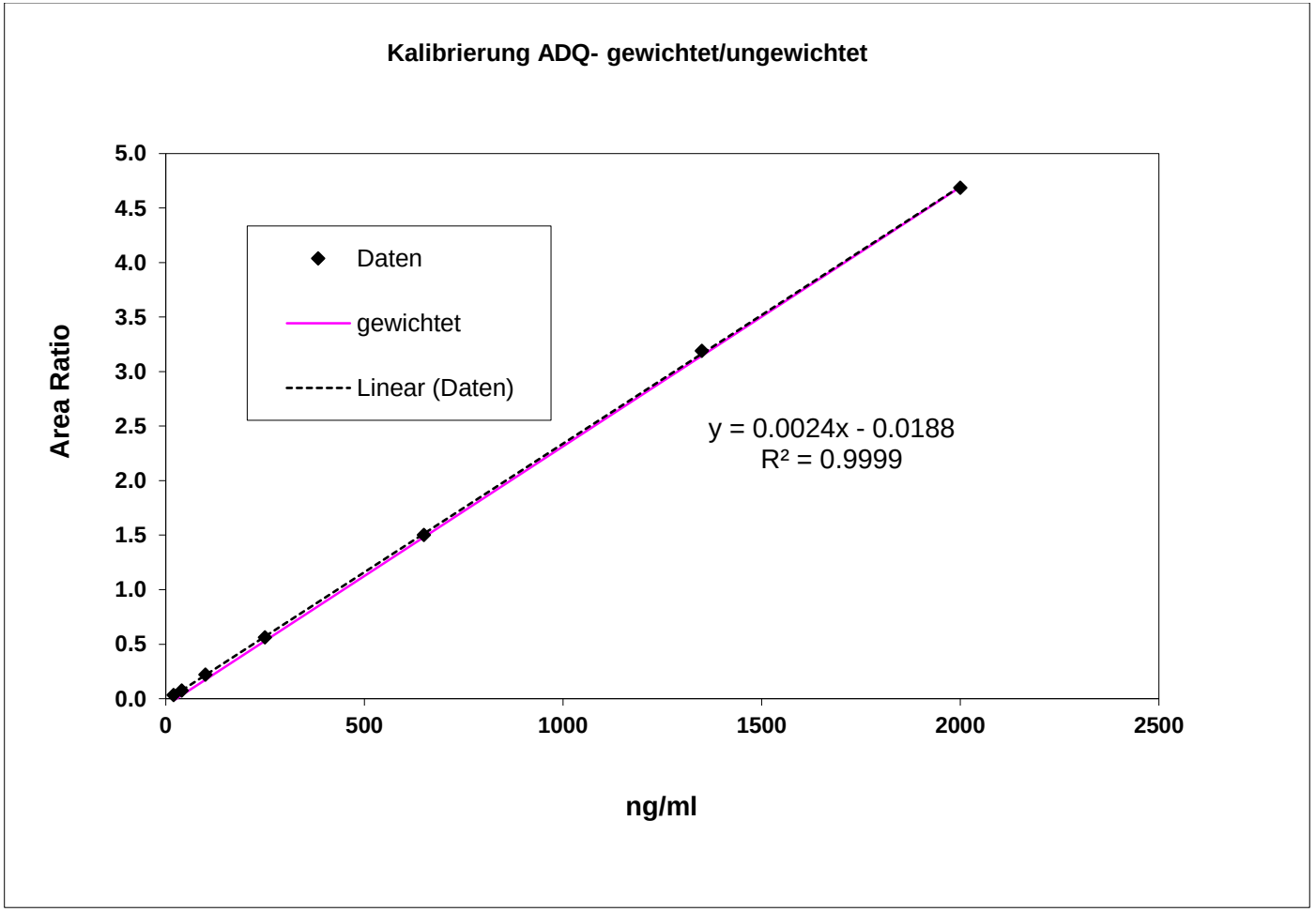
7.0 Appendices

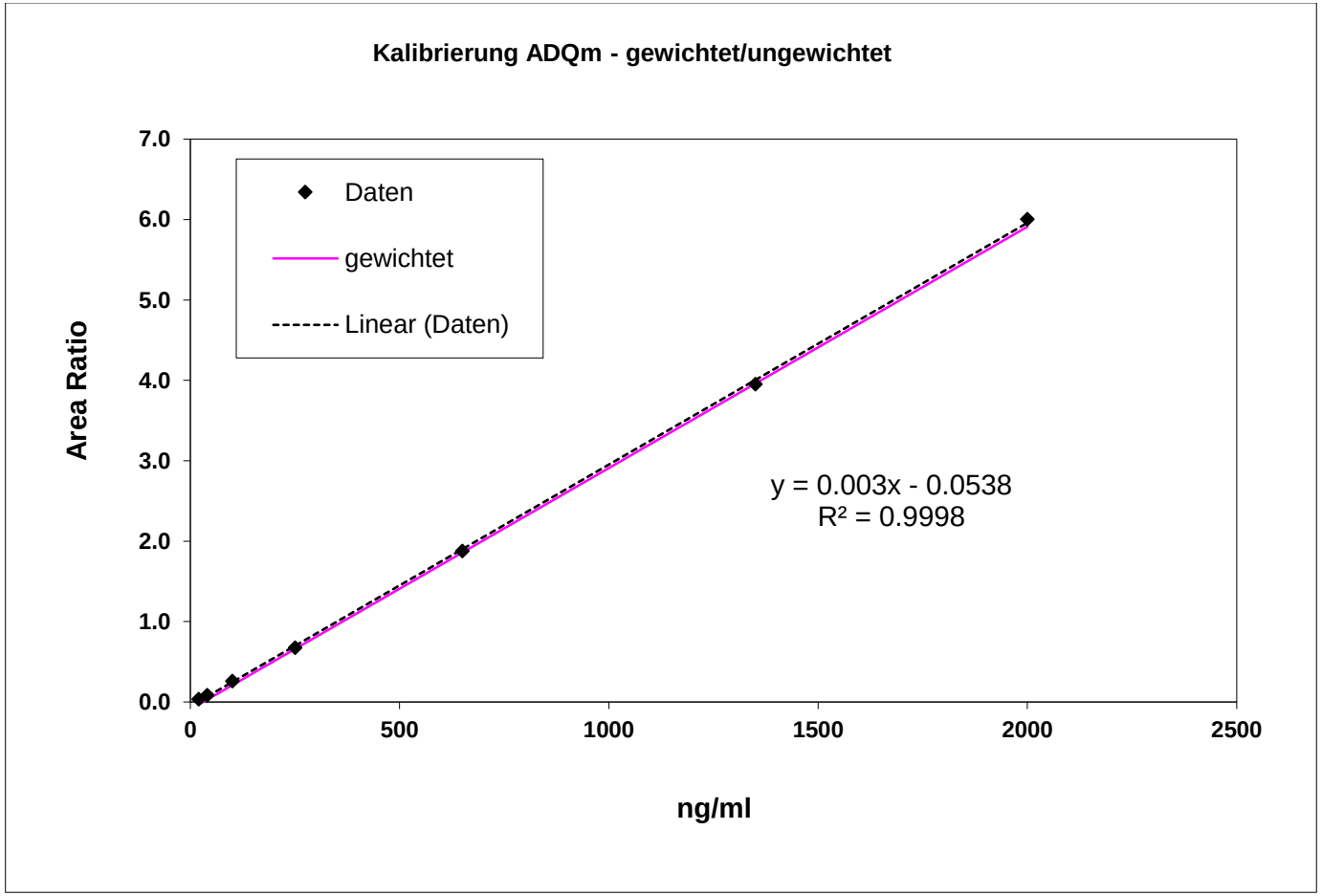
Appendix 1.0 Stages in the life cycle of *Plasmodium falciparum* (Illustration: Coatney GR, Collins WE, Warren M, Contacos PG (1971) The primate malarias. (Bethesda): U.S. Department of Health, Education and Welfare.)



Appendix 2.0 Standard Calibration for Amodiaquine



Appendix 3.0 Standard Calibration for Desethyl-Amodiaquine



Appendix 4.0 Antimalarial drugs used as monotherapy (Modified from Antony et al.,2016)

Antimalarial derivaties	Chemical Family	Drug Name	Half Life	Mechanism of Action	Used	Contraindictions	Status of Resistance	Genetic Markers of Drug Resistance
Quinoline derivates	4-Aminoquinolines	Chloroquine (CQ)	41±14 days	Accumulate in the digestive vacuole of the parasite Inhibition of heme detoxification	Treatment of non-falciparum infections Treatment of falciparum infections where CQ remains sensitive chemoprophylaxis in regions where CQ remains sensitive	-	Yes	Point mutation in Pfcrt, Pfmdr1, Pfmrp
		Amodiaquine (AQ)	5 hrs	Accumulate in the digestive vacuole of parasite Inhibition of heme detoxification	Treatment of non-severe falciparum infections where CQ resistance has emerged	-	Yes	Point mutation and copy number variation in Pfmdr1
	Amino Alchohols	Quinine	10-12 hrs	Accumulate in the digestive vacuole of parasite Inhibition of heme detoxification	Treatment of severe malaria and multidrug-resistance falciparum infections Treatment of malaria during pregnancy in the 1st trimester	-	Yes	Point mutation in Pfmdr1, Pfmrp, Pfnhe1 and copy number variation in Pfmdr1
		Mefloquine	14-18 days	Accumulate in the digestive vacuole of parasite	Treatment of non-severe falciparum infections where CQ and SP resistance has emerged	Known/ suspected history of neuropsychiatric disorder	Yes	Point mutation and copy number variation in Pfmdr1

				Inhibition of heme detoxification	Chemoprophylaxis in regions with CQ resistance	History of seizures		
		Halofantrine	10-90 hrs	Accumulate in the digestive vacuole of parasite Inhibition of heme detoxification	Treatment of suspected multidrug-resistant falciparum malaria infections	Concomitant use of Halofantrine Preexisting cardiac disease Congenital prolongation of QTc interval Treatment of mefloquine within three weeks prior use Pregnancy	Yes	Point mutation and copy number variation in Pfmdr1
		Lumefantrine	3-6 days	Accumulate in the digestive vacuole of parasite Inhibition of heme detoxification	Treatment of non-severe falciparum infections where CQ and SP resistance has emerged		Yes	Point mutation and copy number variation in Pfmdr1
	8-Aminoquinolines	Primaquine	6 hrs	Not precisely known	Treatment of vivax and ovale infections to prevent relapse Gametocytodical agent	G6PD deficiency Pregnancy	Yes	Not known
	Naphtoquinone	Atovaquone	59 hrs	Targets the cytochrome bc1 complex, located in the inner	Treatment of multidrug-resistant		Yes	Point mutation in cytb gene

				mitochondrial membrane of parasite Inhibits the respiratory reaction of parasite	falciparum malaria infections			

Appendix 5.0 Analysis of Variance

1. Lab Strains vs Field Isolates_2009

Tests of Between-Subjects Effects

Dependent Variable: IC50

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	129418.969[a]	21	6162.808	18.208	.000
Intercept	115762.857	1	115762.857	342.030	.000
Drug	49873.632	1	49873.632	147.355	.000
Clones	34652.638	10	3465.264	10.238	.000
Drug * Clones	26125.874	10	2612.587	7.719	.000
Error	28430.516	84	338.459		
Total	310191.579	106			
Corrected Total	157849.485	105			

a. R Squared = .820 (Adjusted R Squared = .775)

Tests of Between-Subjects Effects

Dependent Variable: IC50

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Corrected Model	129418.969[a]	21	6162.808	18.208	.000
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Drug * Clones	26125.874	10	2612.587	7.719	.000
Error	28430.516	84	338.459		
Total	310191.579	106			
Corrected Total	157849.485	105			

a. R Squared = .820 (Adjusted R Squared = .775)

Post Hoc Tests

Clones

Multiple Comparisons

Dependent Variable: IC50

Tukey HSD

(I) Clones	(J) Clones	Mean Difference (I-J)	Std. Error	Sig.	99.9% Confidence Interval Lower Bound	99.9% Confidence Interval Upper Bound
Dd2	GB4	-10.4221	8.51627	.978	-48.8537	28.0094
	HB3	55.5782*	7.41246	.000	22.1279	89.0286
	S16	40.6574*	7.61718	.000	6.2832	75.0316
	S17	47.5031*	8.97694	.000	6.9927	88.0135
	S25	46.7693*	6.95350	.000	15.3901	78.1485
	S27	53.0593*	8.51627	.000	14.6277	91.4908
	S47	28.5694	7.61718	.014	-5.8048	62.9436
	S6	11.8487	7.86016	.913	-23.6221	47.3194
	S79	43.9677*	8.15371	.000	7.1723	80.7631
	S9	15.0714	7.61718	.664	-19.3028	49.4456
GB4	Dd2	10.4221	8.51627	.978	-28.0094	48.8537
	HB3	66.0004*	8.89495	.000	25.8599	106.1408
	S16	51.0796*	9.06626	.000	10.1661	91.9931
	S17	57.9252*	10.23528	.000	11.7363	104.1142
	S25	57.1914*	8.51627	.000	18.7599	95.6230
	S27	63.4814*	9.83374	.000	19.1045	107.8584
	S47	38.9916	9.06626	.002	-1.9219	79.9051
	S6	22.2708	9.27134	.378	-19.5682	64.1098
	S79	54.3898*	9.52148	.000	11.4220	97.3576
	S9	25.4936	9.06626	.172	-15.4199	66.4071
HB3	Dd2	-55.5782*	7.41246	.000	-89.0286	-22.1279
	GB4	-66.0004*	8.89495	.000	-106.1408	-25.8599
	S16	-14.9208	8.03833	.743	-51.1956	21.3539
	S17	-8.0752	9.33695	.999	-50.2102	34.0599
	S25	-8.8090	7.41246	.982	-42.2593	24.6414
	S27	-2.5190	8.89495	1.000	-42.6594	37.6215
	S47	-27.0088	8.03833	.043	-63.2836	9.2659
	S6	-43.7296*	8.26895	.000	-81.0450	-6.4141
	S79	-11.6106	8.54847	.955	-50.1874	26.9663
	S9	-40.5068*	8.03833	.000	-76.7816	-4.2321
S16	Dd2	-40.6574*	7.61718	.000	-75.0316	-6.2832
	GB4	-51.0796*	9.06626	.000	-91.9931	-10.1661

	HB3	14.9208	8.03833	.743	-21.3539	51.1956
	S17	6.8457	9.50030	1.000	-36.0265	49.7179
	S25	6.1119	7.61718	.999	-28.2624	40.4861
	S27	12.4019	9.06626	.953	-28.5116	53.3154
	S47	-12.0880	8.22750	.926	-49.2164	25.0404
	S6	-28.8088	8.45295	.038	-66.9546	9.3370
	S79	3.3102	8.72658	1.000	-36.0704	42.6909
	S9	-25.5860	8.22750	.085	-62.7144	11.5424
S17	Dd2	-47.5031*	8.97694	.000	-88.0135	-6.9927
	GB4	-57.9252*	10.23528	.000	-104.1142	-11.7363
	HB3	8.0752	9.33695	.999	-34.0599	50.2102
	S16	-6.8457	9.50030	1.000	-49.7179	36.0265
	S25	-.7338	8.97694	1.000	-41.2442	39.7766
	S27	5.5562	10.23528	1.000	-40.6328	51.7452
	S47	-18.9337	9.50030	.654	-61.8059	23.9385
	S6	-35.6544	9.69620	.017	-79.4107	8.1018
	S79	-3.5354	9.93565	1.000	-48.3722	41.3014
	S9	-32.4317	9.50030	.037	-75.3039	10.4405
S25	Dd2	-46.7693*	6.95350	.000	-78.1485	-15.3901
	GB4	-57.1914*	8.51627	.000	-95.6230	-18.7599
	HB3	8.8090	7.41246	.982	-24.6414	42.2593
	S16	-6.1119	7.61718	.999	-40.4861	28.2624
	S17	.7338	8.97694	1.000	-39.7766	41.2442
	S27	6.2900	8.51627	1.000	-32.1415	44.7215
	S47	-18.1999	7.61718	.386	-52.5741	16.1744
	S6	-34.9206	7.86016	.001	-70.3913	.5501
	S79	-2.8016	8.15371	1.000	-39.5970	33.9938
	S9	-31.6979	7.61718	.003	-66.0721	2.6764
S27	Dd2	-53.0593*	8.51627	.000	-91.4908	-14.6277
	GB4	-63.4814*	9.83374	.000	-107.8584	-19.1045
	HB3	2.5190	8.89495	1.000	-37.6215	42.6594
	S16	-12.4019	9.06626	.953	-53.3154	28.5116
	S17	-5.5562	10.23528	1.000	-51.7452	40.6328
	S25	-6.2900	8.51627	1.000	-44.7215	32.1415
	S47	-24.4899	9.06626	.217	-65.4034	16.4236
	S6	-41.2106	9.27134	.001	-83.0496	.6283
	S79	-9.0916	9.52148	.997	-52.0594	33.8762
	S9	-37.9879	9.06626	.003	-78.9014	2.9256
S47	Dd2	-28.5694	7.61718	.014	-62.9436	5.8048
	GB4	-38.9916	9.06626	.002	-79.9051	1.9219

	HB3	27.0088	8.03833	.043	-9.2659	63.2836
	S16	12.0880	8.22750	.926	-25.0404	49.2164
	S17	18.9337	9.50030	.654	-23.9385	61.8059
	S25	18.1999	7.61718	.386	-16.1744	52.5741
	S27	24.4899	9.06626	.217	-16.4236	65.4034
	S6	-16.7208	8.45295	.664	-54.8666	21.4250
	S79	15.3982	8.72658	.797	-23.9824	54.7789
	S9	-13.4980	8.22750	.860	-50.6264	23.6304
S6	Dd2	-11.8487	7.86016	.913	-47.3194	23.6221
	GB4	-22.2708	9.27134	.378	-64.1098	19.5682
	HB3	43.7296*	8.26895	.000	6.4141	81.0450
	S16	28.8088	8.45295	.038	-9.3370	66.9546
	S17	35.6544	9.69620	.017	-8.1018	79.4107
	S25	34.9206	7.86016	.001	-.5501	70.3913
	S27	41.2106	9.27134	.001	-.6283	83.0496
	S47	16.7208	8.45295	.664	-21.4250	54.8666
	S79	32.1190	8.93945	.022	-8.2222	72.4603
	S9	3.2228	8.45295	1.000	-34.9230	41.3686
S79	Dd2	-43.9677*	8.15371	.000	-80.7631	-7.1723
	GB4	-54.3898*	9.52148	.000	-97.3576	-11.4220
	HB3	11.6106	8.54847	.955	-26.9663	50.1874
	S16	-3.3102	8.72658	1.000	-42.6909	36.0704
	S17	3.5354	9.93565	1.000	-41.3014	48.3722
	S25	2.8016	8.15371	1.000	-33.9938	39.5970
	S27	9.0916	9.52148	.997	-33.8762	52.0594
	S47	-15.3982	8.72658	.797	-54.7789	23.9824
	S6	-32.1190	8.93945	.022	-72.4603	8.2222
	S9	-28.8962	8.72658	.050	-68.2769	10.4844
S9	Dd2	-15.0714	7.61718	.664	-49.4456	19.3028
	GB4	-25.4936	9.06626	.172	-66.4071	15.4199
	HB3	40.5068*	8.03833	.000	4.2321	76.7816
	S16	25.5860	8.22750	.085	-11.5424	62.7144
	S17	32.4317	9.50030	.037	-10.4405	75.3039
	S25	31.6979	7.61718	.003	-2.6764	66.0721
	S27	37.9879	9.06626	.003	-2.9256	78.9014
	S47	13.4980	8.22750	.860	-23.6304	50.6264
	S6	-3.2228	8.45295	1.000	-41.3686	34.9230
	S79	28.8962	8.72658	.050	-10.4844	68.2769

Based on observed means.

The error term is Mean Square(Error) = 338.459.

*. The mean difference is significant at the .001 level.

2. Clone 9C6

Tests of Between-Subjects Effects

Dependent Variable: logIC50

Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	24.624[a]	35	.704	24.497	.000	.913
Intercept	207.700	1	207.700	7231.901	.000	.989
Clones	3.598	11	.327	11.390	.000	.604
Drug	18.246	2	9.123	317.659	.000	.886
Clones * Drug	1.338	22	.061	2.117	.008	.362
Error	2.355	82	.029			
Total	260.902	118				
Corrected Total	26.979	117				

a. R Squared = .913 (Adjusted R Squared = .875)

a. Clones

Estimates

Dependent Variable: logIC50

Clones	Mean	Std. Error	95% Confidence Interval	
			Lower Bound	Upper Bound
9A4	1.446	.054	1.339	1.554
9A6	1.316	.056	1.204	1.429
9C6	1.521	.054	1.413	1.628
9C9	1.370	.049	1.273	1.467
9D7	1.368	.069	1.231	1.506
9E3	1.197	.069	1.059	1.334
9F1	1.361	.061	1.240	1.482
9F2	1.508	.049	1.410	1.605
9H7	1.472	.050	1.373	1.572
9H8	1.423	.052	1.320	1.525
Dd2	1.620	.050	1.521	1.720
HB3	.911	.054	.804	1.019

Pairwise Comparisons

Dependent Variable: logIC50

(I) Clones	(J) Clones	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
9A4	9A6	.130	.078	.100	-.026	.285
	9C6	-.074	.076	.335	-.226	.078
	9C9	.076	.073	.298	-.069	.222
	9D7	.078	.088	.376	-.097	.253
	9E3	.250*	.088	.006	.075	.424
	9F1	.085	.082	.298	-.077	.248
	9F2	-.061	.073	.402	-.206	.084
	9H7	-.026	.074	.725	-.172	.121
	9H8	.024	.075	.753	-.125	.172
	Dd2	-.174*	.074	.021	-.320	-.027
	HB3	.535*	.076	.000	.383	.687
9A6	9A4	-.130	.078	.100	-.285	.026
	9C6	-.204*	.078	.011	-.360	-.049
	9C9	-.053	.075	.476	-.202	.095
	9D7	-.052	.089	.564	-.229	.126
	9E3	.120	.089	.184	-.058	.297
	9F1	-.045	.083	.594	-.210	.121
	9F2	-.191*	.075	.012	-.340	-.043
	9H7	-.156*	.075	.042	-.306	-.006
	9H8	-.106	.076	.168	-.259	.046
	Dd2	-.304*	.075	.000	-.454	-.154
	HB3	.405*	.078	.000	.249	.561
9C6	9A4	.074	.076	.335	-.078	.226
	9A6	.204*	.078	.011	.049	.360
	9C9	.151*	.073	.042	.006	.296
	9D7	.152	.088	.087	-.022	.327
	9E3	.324*	.088	.000	.149	.498
	9F1	.160	.082	.054	-.003	.322
	9F2	.013	.073	.861	-.132	.158
	9H7	.048	.074	.515	-.098	.195
	9H8	.098	.075	.195	-.051	.246
	Dd2	-.100	.074	.180	-.246	.047
	HB3	.609*	.076	.000	.457	.761
9C9	9A4	-.076	.073	.298	-.222	.069
	9A6	.053	.075	.476	-.095	.202
	9C6	-.151*	.073	.042	-.296	-.006

	9D7	.002	.085	.984	-.167	.170
	9E3	.173*	.085	.044	.005	.342
	9F1	.009	.078	.909	-.147	.165
	9F2	-.138*	.069	.050	-.275	.000
	9H7	-.102	.070	.147	-.242	.037
	9H8	-.053	.071	.459	-.194	.089
	Dd2	-.250*	.070	.001	-.389	-.111
	HB3	.458*	.073	.000	.313	.604
9D7	9A4	-.078	.088	.376	-.253	.097
	9A6	.052	.089	.564	-.126	.229
	9C6	-.152	.088	.087	-.327	.022
	9C9	-.002	.085	.984	-.170	.167
	9E3	.171	.098	.084	-.023	.366
	9F1	.007	.092	.938	-.176	.191
	9F2	-.140	.085	.103	-.308	.029
	9H7	-.104	.085	.226	-.274	.066
	9H8	-.055	.086	.528	-.226	.117
	Dd2	-.252*	.085	.004	-.422	-.082
	HB3	.457*	.088	.000	.282	.631
9E3	9A4	-.250*	.088	.006	-.424	-.075
	9A6	-.120	.089	.184	-.297	.058
	9C6	-.324*	.088	.000	-.498	-.149
	9C9	-.173*	.085	.044	-.342	-.005
	9D7	-.171	.098	.084	-.366	.023
	9F1	-.164	.092	.079	-.348	.019
	9F2	-.311*	.085	.000	-.479	-.142
	9H7	-.275*	.085	.002	-.445	-.106
	9H8	-.226*	.086	.011	-.398	-.054
	Dd2	-.423*	.085	.000	-.593	-.254
	HB3	.285*	.088	.002	.111	.460
9F1	9A4	-.085	.082	.298	-.248	.077
	9A6	.045	.083	.594	-.121	.210
	9C6	-.160	.082	.054	-.322	.003
	9C9	-.009	.078	.909	-.165	.147
	9D7	-.007	.092	.938	-.191	.176
	9E3	.164	.092	.079	-.019	.348
	9F2	-.147	.078	.064	-.302	.009
	9H7	-.111	.079	.162	-.268	.046
	9H8	-.062	.080	.441	-.221	.097
	Dd2	-.259*	.079	.001	-.416	-.102
	HB3	.449*	.082	.000	.287	.612

9F2	9A4	.061	.073	.402	-.084	.206
	9A6	.191*	.075	.012	.043	.340
	9C6	-.013	.073	.861	-.158	.132
	9C9	.138*	.069	.050	.000	.275
	9D7	.140	.085	.103	-.029	.308
	9E3	.311*	.085	.000	.142	.479
	9F1	.147	.078	.064	-.009	.302
	9H7	.035	.070	.614	-.104	.175
	9H8	.085	.071	.236	-.056	.226
	Dd2	-.112	.070	.112	-.252	.027
	HB3	.596*	.073	.000	.451	.741
9H7	9A4	.026	.074	.725	-.121	.172
	9A6	.156*	.075	.042	.006	.306
	9C6	-.048	.074	.515	-.195	.098
	9C9	.102	.070	.147	-.037	.242
	9D7	.104	.085	.226	-.066	.274
	9E3	.275*	.085	.002	.106	.445
	9F1	.111	.079	.162	-.046	.268
	9F2	-.035	.070	.614	-.175	.104
	9H8	.050	.072	.493	-.093	.192
	Dd2	-.148*	.071	.040	-.289	-.007
	HB3	.561*	.074	.000	.414	.707
9H8	9A4	-.024	.075	.753	-.172	.125
	9A6	.106	.076	.168	-.046	.259
	9C6	-.098	.075	.195	-.246	.051
	9C9	.053	.071	.459	-.089	.194
	9D7	.055	.086	.528	-.117	.226
	9E3	.226*	.086	.011	.054	.398
	9F1	.062	.080	.441	-.097	.221
	9F2	-.085	.071	.236	-.226	.056
	9H7	-.050	.072	.493	-.192	.093
	Dd2	-.197*	.072	.007	-.340	-.054
	HB3	.511*	.075	.000	.363	.660
Dd2	9A4	.174*	.074	.021	.027	.320
	9A6	.304*	.075	.000	.154	.454
	9C6	.100	.074	.180	-.047	.246
	9C9	.250*	.070	.001	.111	.389
	9D7	.252*	.085	.004	.082	.422
	9E3	.423*	.085	.000	.254	.593
	9F1	.259*	.079	.001	.102	.416

	9F2	.112	.070	.112	-.027	.252
	9H7	.148*	.071	.040	.007	.289
	9H8	.197*	.072	.007	.054	.340
	HB3	.709*	.074	.000	.562	.855
HB3	9A4	-.535*	.076	.000	-.687	-.383
	9A6	-.405*	.078	.000	-.561	-.249
	9C6	-.609*	.076	.000	-.761	-.457
	9C9	-.458*	.073	.000	-.604	-.313
	9D7	-.457*	.088	.000	-.631	-.282
	9E3	-.285*	.088	.002	-.460	-.111
	9F1	-.449*	.082	.000	-.612	-.287
	9F2	-.596*	.073	.000	-.741	-.451
	9H7	-.561*	.074	.000	-.707	-.414
	9H8	-.511*	.075	.000	-.660	-.363
	Dd2	-.709*	.074	.000	-.855	-.562

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Univariate Tests

Dependent Variable: logIC50

	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Contrast	3.598	11	.327	11.390	.000	.604
Error	2.355	82	.029			

The F tests the effect of Clones. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

b. Drug

Estimates

Dependent Variable: logIC50

			95% Confidence Interval	
Drug	Mean	Std. Error	Lower Bound	Upper Bound
AQ	1.163	.028	1.108	1.219
CQ	1.944	.028	1.889	1.999
DQ	1.021	.029	.965	1.078

Pairwise Comparisons

Dependent Variable: logIC50

(I) Drug	(J) Drug	Mean Difference (I-J)	Std. Error	Sig.[b]	95% Confidence Interval for Difference[b]	
					Lower Bound	Upper Bound
AQ	CQ	-.780*	.039	.000	-.858	-.702
	DQ	.142*	.040	.001	.063	.222
CQ	AQ	.780*	.039	.000	.702	.858
	DQ	.922*	.040	.000	.843	1.001
DQ	AQ	-.142*	.040	.001	-.222	-.063
	CQ	-.922*	.040	.000	-1.001	-.843

Based on estimated marginal means

*. The mean difference is significant at the .05 level.

b. Adjustment for multiple comparisons: Least Significant Difference (equivalent to no adjustments).

Univariate Tests

Dependent Variable: logIC50

	Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Contrast	18.246	2	9.123	317.659	.000	.886
Error	2.355	82	.029			

The F tests the effect of Drug. This test is based on the linearly independent pairwise comparisons among the estimated marginal means.

3. Drug Accumulation Assay

Tests of Between-Subjects Effects

Dependent Variable: Accu

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	19502845.251[a]	19	1026465.540	6.738	.000
Intercept	32821536.932	1	32821536.932	215.457	.000
Clone	8401879.161	4	2100469.790	13.789	.000
Time	4287480.336	3	1429160.112	9.382	.000
Clone * Time	5793017.251	12	482751.438	3.169	.011
Error	3046686.273	20	152334.314		
Total	56870425.456	40			
Corrected Total	22549531.524	39			

a. R Squared = .865 (Adjusted R Squared = .737)

Post Hoc Tests

Clone

Multiple Comparisons

Dependent Variable: Accu

Tukey HSD

(I) Clone	(J) Clone	Mean Difference (I-J)	Std. Error	Sig.	99.9% Confidence Interval Lower Bound	99.9% Confidence Interval Upper Bound
173D3	47C7	860.4600	195.15014	.002	-69.5589	1790.4788
	9C6	1157.2165*	178.14689	.000	308.2293	2006.2037
	Dd2	1278.2610*	195.15014	.000	348.2422	2208.2799
	HB3	776.8080	239.00914	.029	-362.2278	1915.8438
47C7	173D3	-860.4600	195.15014	.002	-1790.4788	69.5589
	9C6	296.7565	178.14689	.476	-552.2306	1145.7437
	Dd2	417.8011	195.15014	.242	-512.2178	1347.8199
	HB3	-83.6520	239.00914	.997	-1222.6878	1055.3838
9C6	173D3	-1157.2165*	178.14689	.000	-2006.2037	-308.2293
	47C7	-296.7565	178.14689	.476	-1145.7437	552.2306
	Dd2	121.0446	178.14689	.959	-727.9426	970.0317
	HB3	-380.4085	225.33998	.463	-1454.3017	693.4848
Dd2	173D3	-1278.2610*	195.15014	.000	-2208.2799	-348.2422
	47C7	-417.8011	195.15014	.242	-1347.8199	512.2178
	9C6	-121.0446	178.14689	.959	-970.0317	727.9426
	HB3	-501.4530	239.00914	.259	-1640.4888	637.5828
HB3	173D3	-776.8080	239.00914	.029	-1915.8438	362.2278
	47C7	83.6520	239.00914	.997	-1055.3838	1222.6878
	9C6	380.4085	225.33998	.463	-693.4848	1454.3017
	Dd2	501.4530	239.00914	.259	-637.5828	1640.4888

Based on observed means.

The error term is Mean Square(Error) = 152334.314.

*. The mean difference is significant at the 0.001 level.

Time

Multiple Comparisons

Dependent Variable: Accu

Tukey HSD

(I) Time	(J) Time	Mean Difference (I-J)	Std. Error	Sig.	99.9% Confidence Interval Lower Bound	99.9% Confidence Interval Upper Bound
20AQ	20CQ	705.7401	174.54759	.003	-90.8298	1502.3099
	5AQ	870.3619*	174.54759	.000	73.7920	1666.9318
	5CQ	896.3632*	174.54759	.000	99.7934	1692.9331
20CQ	20AQ	-705.7401	174.54759	.003	-1502.3099	90.8298
	5AQ	164.6218	174.54759	.782	-631.9480	961.1917
	5CQ	190.6232	174.54759	.698	-605.9467	987.1930
5AQ	20AQ	-870.3619*	174.54759	.000	-1666.9318	-73.7920
	20CQ	-164.6218	174.54759	.782	-961.1917	631.9480
	5CQ	26.0013	174.54759	.999	-770.5685	822.5712
5CQ	20AQ	-896.3632*	174.54759	.000	-1692.9331	-99.7934
	20CQ	-190.6232	174.54759	.698	-987.1930	605.9467
	5AQ	-26.0013	174.54759	.999	-822.5712	770.5685

Based on observed means.

The error term is Mean Square(Error) = 152334.314.

*. The mean difference is significant at the 0.001 level.

4. Drug Accumulation Assay (Adapted strains vs Recent strains)

Tests of Between-Subjects Effects

Dependent Variable: Uptake

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2011580.561[a]	5	402316.112	1.226	.340
Intercept	12571935.461	1	12571935.461	38.306	.000
Clone	1217497.230	2	608748.615	1.855	.187
New_Old	11579.377	1	11579.377	.035	.853
Clone * New_Old	770575.464	2	385287.732	1.174	.333
Error	5579418.942	17	328201.114		
Total	20685200.347	23			
Corrected Total	7590999.503	22			

a. R Squared = .265 (Adjusted R Squared = .049)

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
Uptake	.268	23	.000	.868	23	.006

a. Lilliefors Significance Correction

b. AQ-20 mins

Tests of Between-Subjects Effects

Dependent Variable: Uptake

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	5216954.248[a]	5	1043390.850	1.519	.231
Intercept	42265804.042	1	42265804.042	61.519	.000
New_Old	1587351.566	1	1587351.566	2.310	.145
Clone	3301111.233	2	1650555.617	2.402	.117
New_Old * Clone	32952.952	2	16476.476	.024	.976
Error	13053741.505	19	687039.027		
Total	64032587.021	25			
Corrected Total	18270695.752	24			

a. R Squared = .286 (Adjusted R Squared = .098)