GENETIC POLYMORPHISMS IN *PLASMODIUM FALCIPARUM* ASSOCIATED WITH RESISTANCE TO ARTEMISININ COMBINATION THERAPY: COMPLEXITY OF INFECTION DURING SHORT-TERM CULTURE AND CHARACTERIZATION OF *IN VITRO* SENSITIVITY IN KAMPALA UGANDA

BY

NSOBYA SAMUEL LUBWAMA.

BLT.DipMLT, Msc.Mol.Bio.

2006/HD11/4547U

THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES FOR AWARD OF DEGREE OF DOCTOR OF PHILOSOPHY OF MAKERERE UNIVERSITY

SUPERVISORS

Professor Philip Rosenthal. M.D	**University of California, San Francisco**
Assoc. Professor Moses Joloba MBChB. Msc. PhD	**Makerere University Kampala**

DECLARATION

I Nsobya Sammuel Lubwama do declare that this work is my own and original, and has never been published and submitted for any other degree award to any other university.

Signature…….. Date……..

This thsis has been submitted for examination with the approval of the following supervisors:

Professor Philip Rosenthal. M.D Sign…… Date……..

Department of Medicine

San Francisco General Hospital

University of California, San Francisco

E- mail: prosenthal@medsfgh.ucsf

Assoc. Professor Moses Joloba MBChB. Msc. PhD Sign…… Date……

Department of Microbiology

Makerere University

E- mail: m.joloba@gmail.com

DEDICATION

In loving memory of Late Robinson Nanyenya and Mrs Samali Nanyenya

And

My Wife Edith Nsobya, Children; Brenda, Andrew, Gordon, Jovan and Benita

ACKNOWLEDGEMENT

1 would like to gratefully acknowledge the enthusiastic supervision of Professor Philip J Rosenthal, Assoc. Professor Moses Joloba during this work. I thank Dr. Harald Noedl, Medical University of Vienna, for valuable advice regarding *in vitro* drug sensitivity assays with numerous stimulating discussions through, e- mail from Vienna which helped me a lot in trouble shooting with experimental setup. Malaria Research and Reference Reagent Resource Center for free reference control *P. falciparum* strains. Norvatis for free artemsinin derivatives. 1 also thank the participants in the clinical trial from Tororo and Kampala, their parents and guardians, and our clinical study team from MU/UCSF and UMSP for there input. Further, I am indebted to my wife and children for their understanding, endless patience and encouragement for the time l have been away.

Finally I acknowledge the support of this PhD work from the following grants: the National Institutes of Health/ Fogarty International Center (KO1 TW00007), Centers for Disease Control and Malaria Surveillance and Control in Uganda (SA3569 and S1932-21/21) and the Doris Duke Charitable Foundation. Professor Philip J Rosenthal is a Doris Duke Charitable Foundation Distinguished Clinical Scientist and Dr Grant Dorsey is the recipient of a DDCF Clinical Scientist Development Award.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ACT	Artemisinin-based combination therapy
AL	artemether-lumefantrine
APAD	*3-acetyl pyridine adenine dinucleotide*
Artekin	dihydroartemisinin and piperaquine
AS/AQ	artesunate-amodiaquine
ASRA	PCR-allele specific restriction analysis
Coartem	artemether-lumefantrine
COI	complexity of infection
CQ	Chloroquine
CQR	Chloroquine resistance
CV4	dihydroartemisinin, trimethoprim, piperaquine phosphate and primaquine phosphate
MDAQ	Monodesetylamodiaquine
DELI test	double-site enzyme-linked LDH immunodetection
DHA	Dihydroartemisinin
dhfr	dihydrofolate reductase
dhps	dihydropteroate synthase
DNA	Deoxyribonucleic acid
Duo-Cotecxin	dihydroartemisinin and piperaquine
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
Hepes Buffer	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP2	*Histidine-rich protein 2*
IC50	50% inhibition concentration
LUM	Lumefrantrine
MOLAB	Molecular Research Laboratory
MPFG-45	antibody conjugated to hoseradish peroxidase
MPFM-45A	IgM capture antibody specific for *P. falciparum HRP2*
msp-2	*merozoite surface protein 2*
MU – UCSF	Makerere University/ University of California San Fransisco
NAD	*nicotinamide adenine dinucleotide*
NaHCO3	*Sodium Bicarbonate*
PBS	phoshate buffer saline
PCR	polymerase chain reaction
pfatpase6	*sarco/endoplasmic reticulum Ca2+-ATPase (SERCA), (calcium transporter gene*
pfcrt	*Plasmodium falciparum chloroquine resistant gene*

pfmdr1	*P. falciparum multidrug resistance gene*
pfnhe1	*Plasmodium falciparum Na+/H+ exchanger gene*
Pgh-1	*P glycoprotein homoloque*
PIP	Piperaquine
Pldh	*Lactate dehydrogenase*
QN	Quinine
RBC	Red blood cells
RPMI 1640	Roswell Park Memorial Institute medium
SNPs	Single nuclear polymorphisms
SP	sulfadoxine and pyrimethamine
SPSS	Computer program used for statistical analysis
STATA	General-purpose statistical software
SYBR Green 1	Dye with marked fluorescence enhancement upon contact with Plasmodium DNA
WBC	White blood cells
WHO	World Health Organization

ABSTRACT

Background

Artemisinin-based combination therapy (ACT) is currently advocated in Africa as a means of improving treatment efficacy and slowing the development of drug resistance. However, the selection of resistant parasites, particularly to artemisinin partner drugs, remains a concern.

We describe a series of studies focusing on molecular determinants of *Plasmodium falciparum* resistance to antimalarial drugs and their correlation with the *in vitro* susceptibility to key antimalarials in practice. This work was also extended to address the important questions about the polyclonal diversity of patient isolates as they were adapted to in vitro culture.

Objectives

i). Assessed changes in occurrence of various polymorphisms of a drug resistant allele's of *pfmdr-1* and *pfcrt* between baseline and new infections during therapy with artesunate-amodiaquine (AS/AQ). ii). Evaluated changes in complexity of infection (COI) during culture. iii). Assessed the impact of various amodiaquine containing regimens on the sensitivity of recurrent *P. falciparum* isolates. iv). Determined *in vitro senstivity* patterns of *P. falciparum* isolates in Uganda against various antimalarial drugs. v). Evaluated associations between parasite genetic polymorphisms, *in vitro drug* sensitivity, and clinical outcomes after antimalarial combination therapy.

Methods

Polymorphisms of the *pfmdr-1* and *pfcrt* genes known to play a role in altered drug sensitivity to some antimalarials were analysed by nested PCR and restriction fragment length polymorphism techniques. Complexity of infection *Plasmodium falciparum* isolates were determined based on *msp-2* polymorphisms using nested PCR. For the *in vitro* sensitivity studies, IC_{50}'s for *P. falciparum* isolates were measured using an *HRP-2-based ELISA.*

Results

a).Treatment with the combination of (AS/AQ) resulted in significant enrichment of isolates harboring the N86Y and D1246Y mutations, and the wild type residueY184, in the *pfmdr1*gene. b). Using the *msp-2* gene marker, isolates were found to lose certain genotypes *in vitro* during culture adaptation, leading to a reduced complexity of infection (COI). Some isolates were also found to harbor new genotypes that appeared after day 0, suggesting that in some cases parasites classified as new infections post-treatment were actually present at the time of treatment initiation and would therefore be misclassified. c). Antimalarial regimens containing amodiaquine were associated with subsequent infections displaying a reduced in vitro susceptibility (although this was not reflected in higher rates of treatment failure). d). Patterns of cross resistance, or inverse susceptibility, were observed between several antimalarial drugs.

Conclusion

Novelty of this study is the analysis of changes of complexity of infection during isolate adaptation to *in vitro* culture, and evidence for decreased susceptibility to amodiaquine pending treatment with regimens containing this drug (though these changes did not lead to reduced clinical efficacy).The study also provides important baseline data to further evaluate the *in vitro* susceptibility of parasite strains in Kampala Uganda and assess any signs of emerging resistance. Lastly, provides useful information on the prevalence of mutations in *pfcrt* and *pfmdrI* and their association with elevated drugsIC_{50} values *in vitro*.

CHAPTER ONE

INTRODUCTION

Malaria is still a major public health problem, and the disease kills around 1.5 million people annually worldwide, mostly in Africa (WHO). Increasing drug resistance has necessitated changes in antimalarial therapy in Africa (Greenwood and others 2005). In Uganda as well as other countries, malaria treatment and control are hindered by the spread of resistance to common antimalarial drugs. This has led WHO to recommend the use of artemisinin-based combination therapy (ACT), which is highly efficacious. Multiple highly effective ACT regimens are now available, but the optimal choice for malaria in most areas remains uncertain. Artemisinin and its derivatives are the most potent and rapidly acting antimalarials. However, artemisinin resistance has been reported in murine models of malaria(Ferrer-Rodriguez and others 2004). In addition, although ACTs have excellent treatment efficacy, monitoring the efficacy of individual components in drug combinations and other antimalarials by *in vitro* drug senstivity assay is still important, particularly in Uganda, where there are no published data regarding *in vitro* antimalarial sensitivity.

Techniques to culture erythrocytic stages of *Plasmodium falciparum* have been available for over 30 years (Trager and Jensen 1976). The study of culture-adapted strains is essential for much laboratory research on malaria; these strains have typically been cultured for months to years before analysis. Culture of fresh parasites is used to study the *in vitro* drug sensitivity of infecting parasites and to search for associations between parasite features and clinical outcomes.

In vitro drug senstivity assays provide information on the natural response of *Plasmodium falciparum* to antimalarials free from influences of host immunity. Fresh clinical isolates are

used in *in vitro* antimalarial susceptibility studies to evaluate the activity of the drugs. Limited studies have explored the effects of ACTs on the drug sensitivity of fresh clinical isolates or on parasite genotypes. Consequently, parasite culture and molecular techniques have been used to investigate the possibility of altered drug sensitivity and specific genotypes being selected after exposure to different recommended ACTs. In addition, there was no published data showing changes in complexity of *Plasmodium falciparum* genotypes as they are being adapted to continuous culture. We investigated whether unique strains that are more fit are being selected which may result in alteration of *in vitro* drug sensitivity.

Molecular monitoring techniques are being developed to complement *in vitro* surveillance. Assessing polymorphisms in candidate drug resistance genes is of outmost relevance to establish their role in modulating drug responses. Although ACTs are highly efficacious, recent clinical studies in Uganda have shown that plasmodium strains with mutations in genes already associated with resistance are being selected by ACT therapy. However data associating *in vitro drug* sensitivity, genotypes and treatment outcome for most recommended antimalarials is still lacking in Uganda.

1.1 Research problem

ACTs have become the standard for treatment of malaria in sub-Saharan Africa. All ACTs combine a short-acting artemisinin with a long acting partner drug, and continued success of these regimens depends on activity of both component drugs. Prolonged body circulation of artemisinin partner drugs suggests that selection of resistance to these agents may occur readily. Thus, the need for regular and comprehensive surveillance including molecular tests, of resistance is a high priority. Surveillance should ideally include *in vitro* and in vivo drug efficacy assays and the monitoring of molecular markers associated with resistance to the

components of ACTs. *In vitro drug* senstivity data will help characterize the large picture of drug resistance in Uganda. In addition *P. falciparum* infections are commonly polyclonal in many areas, and since not all parasites successfully grow in culture, it is unclear how well *in vitro* culture represents the complexity of clinical infections. Further, the roles of different mutations in the *pfcrt* and *pfmdr-1* genes in mediating resistance to artemisinins and long-acting artemisinin partner drugs remains unclear. In particular, associations between parasite genotypes, *in vitro* drug sensitivity, and treatment outcomes for many new antimalarial combinations have been inadequately characterized in Africa, and thus there is a need for further studies in this area.

CHAPTER TWO

LITERATURE REVIEW

2.1 Antimalarial drugs

Quinolines have been the most important class of antimalarial drugs. Quinoline - containing antimalarial drugs chloroquine(CQ), quinine(QN), amodiaquine(AQ) and mefloquine (MEF) are used widely to treat malaria; however the malarial parasites are rapidly becoming resistant. Chloroquine, a 4-aminoquinoline compound, is easily synthesized, cheap, non-toxic and generally well tolerated. It was introduced in 1944–1945 and soon became the mainstay of therapy and prevention, but currently it is no longer effective in most parts of the world because of CQ resistance. The precise mode of action of CQ is not known, but it is generally believed that the drug alters parasite hemoglobin metabolism. When the malaria parasite is in the stage of intraerythrocytic development and proliferation, hemoglobin is a major source of nutrition, and is transported into the acidic food vacuole and sequentially digested into peptide fragments by aspartic, cysteine, and metallo proteases, with heme as by product. Free heme is toxic, and CQ blocks the sequestration of heme into hemozin, thereby killing the parasites.

Amodiaquine is chemically related to CQ, but is more effective than CQ for clearing parasitemia in cases of uncomplicated malaria, even against some CQ-resistant strains (O'Neill and others 1998) and (Ringwald and others 1996). Although drug resistance and potential hepatic toxicity limit its use, it still remains useful for treatment of malaria in many parts of Africa. It has been shown to bind heme and to inhibit heme polymerization *in vitro*, with a similar efficiency to that of CQ (Foley and Tilley 1998). Amodiaquine remains effective in areas of substantial CQ resistance (Staedke and others 2001) and (Gorissen and others 2000). Importantly, AQ is a leading artemisinin partner drug, in the recently released coformulated compound AS/AQ. Mechanisms of resistance to AQ are incompletely understood. The

mechanism is likely similar to that of CQ, as cross-resistance is well described(Olliaro and others 1996) and CQ resistant strains displayed reduced accumulation of AQ (Bray and others 1996b). However, AQ is active against many CQ-resistant parasites(Olliaro and others 1996), and its superiority is demonstrated by the markedly improved antimalarial efficacy of AQ/SP over that of CQ/SP (Staedke and others 2004).

Quinine a quinoline-methanol isolated from the bark of Peruvian cinchona trees, was introduced into Europe from South America in the 17th century, and was the mainstay of malaria chemotherapy until after the Second World War. It remains an essential antimalarial drug for severe *P. falciparum* malaria and intravenous infusion. Quinine interacts weakly with heme, but has been shown to inhibit heme polymerization *in vitro*. The mechanism of resistance to quinine is unknown. Recently, quantitative trait locus analysis identified variations in the pfnhe-1 gene, in addition to pfcrt and pfmdr1, as potentially mediating quinine sensitivity ((Reed and others 2000),(Sidhu and others 2002), (Ferdig and others 2004) and (Nkrumah and others 2009).

Lumefantrine (benflumetol, *alpha- dibutylaminomethyl 2, 7-dichloro-9- chlorobenylidene-fluorenemethanol*) is closely related to halofantrine, a drug that offers potent antimalarial activity, but is limited by serious cardiac toxicity. In its biological activity it resembles the class-2 aryl amino-alcohols, in which the quinoline portion of the 4-quinolinemethanols is replaced by a different aromatic ring system. It is a highly lipophilic compound. Lumefantrine in combination with artemether show synergistic activity with high potency and this combination, (Coartem), is currently recommended as the first line treatment of uncomplicated malaria in many African countries, including Uganda.

Piperaquine(PIP) is a bisquinoline with two quinoline nuclei bound by a covalent aliphatic chain. It was identified as a promising candidate during drug screening programs in the 1960s. It was then used heavily as monotherapy for falciparum malaria. in China, followed by the development of widespread drug resistance (Davis and others 2005), Piperaquine is a highly lipid-soluble drug, with a large volume of distribution,with long elimination half-life and a clearance that is markedly higher in children than in adults. The tolerability, efficacy, pharmacokinetic profile and low cost of piperaquine make it a promising partner drug for use as part of ACT. Piperaquine was re-evaluated in the 1990's and shown to be active *in vitro* against chloroquine-resistant *P. falciparum* isolates.

It was therefore deemed suitable for combination with artemisinin derivatives. Piperaquine-based ACT began as China-Vietnam 4 (CV4): dihydroartemisinin [DHA], trimethoprim, piperaquine phosphate and primaquine phosphate), which was followed by CV8 (the same components as CV4 but in increased quantities), Artecom (in which primaquine was omitted) and Artekin or Duo-Cotecxin(DHA and piperaquine phosphate only). DHA/piperaquine has recently shown outstanding efficacy against uncomplicated malaria in Eastern Uganda (Kamya and others 2007) and Bukina Faso (Zongo and others 2007a).

2.2 Antifolates

Antifolates are the antimalarial drugs currently in use with the best-defined molecular targets. They are subdivided into two classes depending on the enzymes they inhibit in folate metabolism pathway. Sulfa drugs and dapsone inhibit *dihydropteroate synthase* (*dhps*). Proguanil and pyrimethamine act on *dihydrofolate reductase* (*dhfr*). Antifolates attack all growing stages of malaria parasites and are found to inhibit the early growing stages in the liver and the developing infective stages in the mosquito. Useful antifolate drugs for treatment of

malaria are synergistic mixtures of inhibitors of *dhfr* and *dhps.* Available combinations used in the treatment of malaria include: pyrimethamine/sulfadoxine, sulfalene/pyrimethamine and dapsone/pyrimethamine.

2.3 Artemisinins

Artemisinin (*qinghaosu*), a sesquiterpene trioxane lactone, was isolated in 1971 from *Artemisia annua* (sweet or annual wormwood plant) as the active component responsible for antimalarial activity. Artemisinin and its derivatives represent a new class of antimalarials containing an endoperoxide moiety. Several semi-synthetic artemisinin derivatives with better pharmacokinetic parameters have been produced chemically by modifying the parent compound at the C10 position to create the following compounds: dihydroartemisinin, sodium artesunate, artemether, arteether, and artelinic acid (Woodrow and others 2005). These compounds are more potent than artemisinin, but have short plasma half-lives. The replacement of oxygen at the C-10 position of dihydroartemisinin (DHA) with carbon produces a compounds not only with greater hydrolytic stability but also with longer half-life and lower toxicity (O'Neill and Posner 2004). All artemisinins are readily metabolized to the biologically active metabolite DHA. Artemisinin and artemisinin derivatives kill all stages of plasmodium species that infect humans, possibly by interacting with heme to produce carbon- centered free radicals that alkylate protein and damage the organelles and membranes of the parasite. A recent alternative hypothesis is that *PFATPase6* codes for sarco/endoplasmic reticulum Ca^{2+}-*ATPase (SERCA*), (calcium transporter), an ortholog of the mammalian *SERCA*, is a major chemotherapeutic target of artemisinin derivatives(Eckstein-Ludwig and others 2003). Recent studies suggest artemisinin may inhibit *ATPase* and alter intracellular calcium stores (Eckstein-

Ludwig and others 2003). The major drawback of artemisinin derivatives is their short half-life (3–5hrs). For this reason artemisinins are used in combination with other antimalarial agents.

2.4 Molecular determinants of antimalarial drug resistance

These are useful tools that complement phenotypic assays for drug resistance. They also guide the design of strategies to avoid such resistance once it has reached levels of clinical significance. Molecular markers theoretically offer the earliest way to detect emerging drug resistance and intervene accordingly, since they examine fundamental processes in the resistance pathway.

2.5 Plasmodium falciparum chloroquine resistant gene (*pfcrt*)

The *pfcrt* gene, located on chromosome 7, has 13 exons and encodes an integral membrane protein, located on the membrane of the intra-erythrocytic parasite's digestive vacuole. It is a member of a drug/metabolite transporter super family (Martin and Kirk 2004; Tran and Saier 2004). Twenty point mutations in the *pfcrt* gene to date have been associated with chloroquine resistance in the field (Cooper and others 2005). These association studies have shown that substitution of threonine (T) for lysine (K) at position 76 (*K76T*) is the hallmark of chloroquine resistance(CQR) in parasites worldwide((Sidhu and others 2002), (Djimde and others 2001), (Reed and others 2000), (Valderramos and Fidock 2006) and(Bray and others 2005) Additional studies (Durand and others 2001; Johnson and others 2004; Sarr and others 2005; Thomas and others 2002; Vinayak and others 2003) reported few exceptions, with parasites with the 76T allele of *pfcrt* cleared by CQ treatment or those with the *76K* allele leading to resistance, but it was suggested that these results could be due to inaccurate drug tests in the field, mixed infections in vivo or new compensatory mutations in vitro such as *S163R*, which may counteract the *K76T* substitution. However, in one study done in Uganda, where chloroquine

resistance is prevalent, 100% prevalence of the 76T mutation was seen (Dorsey and others 2001). The 76T mutation with other specific single nucleotide polymorphisms (SNPs) at positions 72 –76 form haplotypes that have been associated with geographically diverse chloroquine resistant strains. The two haplotypes, CVIET from Asian and African isolates and SVMNT from South America have been associated with CQ resistance (Cortese and others 2002; Vieira and others 2004; Voruganti and others 2002). Additional studies (Dittrich and others 2005; Warhurst 2003) reported that the SVMNT haplotype found in Southeast Asia appeared to be associated with a decreased efficacy of amodiaquine (AQ).

pfcrt is a putative multiple drug transporter, as implied from CQ resistance studies (Johnson and others 2004) and bioinformatic predictions (Martin and Kirk 2004; Tran and Saier 2004)**.***pfcrt* polymorphisms may also play a role in parasite response to other antimalarial drugs. Interestingly, substitutions at position 76 (*K76I* or *K76N*) not only confer greater resistance to CQ and AQ, but also render the parasite more sensitive to quinine, mefloquine, halofantrine and artemisinin(Cooper and others 2002).

2.6 Plasmodium falciparum multidrug resistance gene (*pfmdr-1*)

The *pfmdr-1* gene, located on chromosome 5, encodes a predicted 12-transmembrane domain protein, known as *P glycoprotein homoloque* (*Pgh-1*) a member of the *ABC* transporter family (Foote and others 1989) (Wilson and others 1989). *Pfmdr-1* is a homologue of mammalian *P glycoprotein* and shares common features which are the determinants of multi drug resistance in mammalian tumor cells (Duraisingh and Cowman 2005). The *P glycoprotein homoloque* is located on the parasite digestive vacuole, which is the site of action of CQ and possibly of other quinoline-based antimalarial drugs, including AQ and QN (Foley and Tilley 1998) and (O'Neill and others 1998). Five different mutations in *pfmdr-1* have been associated both in vivo and *in*

vitro with drug resistance to quinine, halofantrine and artemisinin derivatives: *N86Y, F184Y, S1034C, N1042D,* and *D1246Y* (Wongsrichanalai and others 2002) (Price and others 1999) (Duraisingh and others 2000b). Recent studies (Dokomajilar and others 2006b; Sisowath and others 2005) have also reported that wild type *pfmdr-1 86N* is selected for by prior therapy with AL, There is need to evaluate the above mutations and see how they are linked to resistance in parasites that have been exposed to antimalarial drugs in areas with high endemicity like Uganda.

2.7 Plasmodium falciparum multi drug resistance copy number

*Pfmdr-1*gene amplification (increased copy number) and expression have also been correlated with parasite responses to mefloquine, artemisinins, quinine and lumefantrine (Foote and others 1989) (Price and others 2006) (Pickard and others 2003)and (Price and others 2004), but not to CQ or AQ. The evidence indicating that *pfmdr-1* copy number reduces *in vitro* senstivity to the above antimalarials emphasizes the importance of monitoring this marker. However, to date alterations in *pfmdr-1* copy number, which are common in parts of Asia, have rarely been reported in Africa.

2.8 *Pfmdr-1* and *pfcrt* gene interactions

Although *pfcrt* and *pfmdr-1* are located on chromosomes 7 and 5, respectively, several recent studies have found a strong association between polymorphisms in the two genes (Babiker and others 2001; Ferdig and others 2004; Holmgren and others 2006; Khalil and others 2005; Mu and others 2003). The most important associations have been found between *pfmdr-1 N86Y* and *pfcrt K76T* in field isolates by (Adagut and Warhurst 2001; Anderson and Roper 2005; Babiker and others 2001; Djimde and others 2001; Holmgren and others 2006; Mockenhaupt and others 2005). SNPs in *pfcrt* and *pfmdr-1* genes as well as changes in copy number tend to affect

parasite sensitivity to quinine, mefloquine and artemisinin while often inversely affecting sensitivity of chloroquine (Price and others 2004). In addition, a recent study (Ferdig and others 2004) identified specific regions in *pfmdr-1,pfcrt* and *pfnhe1* on chromosomes 5,7 and 13 respectively as possible modulators of quinine resistance, but this observation needs additional validation.

2.9 *dhfr* and *dhps* genes

These genes encode *P. falciparum* enzymes which are targets for antifolates. In *P. falciparum* SP resistance is associated with point mutations appearing in dihydrofolate reductase (*pf-dhfr*) and dihydropteroate synthase (*pf-dhps*) genes(Nzila-Mounda and others 1998; Triglia and others 1997). In *pf-dhfr,* point mutations changing *Asn51* to *Ile* (*N51I*), *Cys59* to *Arg(C59R*), *Ser108* to *Asn(S108N*), and *Ile164* to *Leu(I164L*) have been shown to confer resistance to pyrimethamine.(Gregson and Plowe 2005) with *S108N* usually observed first, and parasites that carry *pf-dhfr* alleles with mutations at *N51I a*nd/or *C59R* and *I164L*, resulting in double, triple, or quadruple mutants, increasingly resistant to pyrimethamine. Sulfadoxine resistance depends on the following *dhps* mutations: codons 436(*S436A/F), 437(A437G), 540(K540E), 581(A581G),* and *613*(*A613S/T*)(Triglia and Cowman 1994; Wang and others 1997).

2.10 Pfatpase6 gene

PFATPase6 codes for sarco/endoplasmic reticulum Ca^{2+}*-ATPase (SERCA*), (calcium transporter), an ortholog of the mammalian *SERCA*. It was recently hypothesized that it is a major chemotherapeutic target of artemisinin derivatives(Eckstein-Ludwig and others 2003). Recent studies suggest artemisinin may inhibit *ATPase* and alter intracellular calcium stores (Eckstein-Ludwig and others 2003). It was reported (Price and others 2004) that mutation *I89T* in *PFATPase6* has been seen in isolates with a wide range of senstivities to artemsinins. In

addition, (Jambou and others 2005) reported that one particular polymorphism, *PFATPase6 S769N*, correlated significantly with decreased *in vitro* sensitivity to artemether in parasites from French Guiana. These results have important implications for assessment of artemisinin resistance in field isolates, but whether *PfATPase6* plays a role in this process remains unclear

2.11 Pfnhe1 gene

Pfnhe1 located on chromosome13, contains a predicted Na+/H+ exchanger. It is one of the recent implicated putative transporters in modulating parasite response to antimalarials (Anderson and others 2005; Ferdig and others 2004; Mu and others 2003). Analysis of microsatellite variations noted a significant association between DNNND repeats in the C-terminal cytoplasmic domain of pfnhe1 and *in vitro* response to QN. Considering clinical isolates, alterations in numbers of two microsatellite repeats in *pfnhe1* were associated with altered in vitro sensitivity to QN(Nkrumah and others 2009). Engineered parasites with decreased expression of *pfnhe1* had decreased QN sensitivity(Nkrumah and others 2009). However, associations between *pfnhe1* variation and QN resistance may be strain-specific, and the relevance of *pfnhe1* variation in clinical responses to QN and related drugs has not yet been reported.

2.12 Other genes

Multiple other genes have been associated with resistance. Recently it has been predicted that multiple transporters contribute to different phenotypes of resistance (Mu and others 2003). A putative ATP-binding cassette transporter (the same family as *pfmdr1*) is encoded by *pfmrp1*. Two mutations in *pfmrp1* were associated with decreased sensitivity to CQ and QN in field isolates (Mu and others 2003) although other studies failed to see this association(Anderson

and others 2005) and(Cojean and others 2006). Recently, knockout of *pfmrp* was shown to increase parasite accumulation of CQ and QN and to increase sensitivity to these and other drugs(Raj and others 2009) Taken together, recent results suggest that resistance to arylaminoalcohols is complex, with contributions of multiple genes, and that characterization of multiple genes will be needed to characterize this trait One recent study (Anderson and Roper 2005) reported that transporter gene *G7* may be associated with artemisinin response and encouraged further studies for replica results.

2.13 Artemisinin-based combination therapies (ACTs)

Artemisinin-based combination therapies (ACTs) are now recommended by the WHO as first-line treatment for uncomplicated *P.falciparum* malaria in most countries where resistance has compromised the efficacy of older drugs. Current ACT regimens include: artemether/lumefantrine, artesunate/amodiaquine, artesunate/mefloquine, sulfadoxine/pyrimethamine-artesunate and dihydroartemisinin/piperaquine. ACTs are costly, but highly efficacious, benefiting from the very rapid reductions in parasite biomass afforded by artemisinin derivatives with short plasma elimination half-lives, combined with a longer-acting partner drug(Ashley and White 2005). ACTs are being evaluated currently in many clinical trials in Africa. Recent studies have shown some recrudescences and, in high transmission regions, high rates of reinfection after ACT. Briefly, studies by our group in Uganda reported recrudescence rates after therapy with ACT regimens ranging from 1- 12% after 28 day follow up (Dorsey and others 2007) (Yeka and others 2005) (Dorsey and others 2002) (Staedke and others 2004). A recent study done in Tororo comparing two arms, Coartem vs. AQ/AS reported recrudesce rates of 8.4% and 4.2% respectively, and new infections within 28 days of treatment in 66% and 55% of subjects.(Bukirwa and others 2006). In addition, in the same study

population, (Dokomajilar and others 2006b) identified independent selection of three polymorphisms associated with diminished response to lumefantrine in the *pfmdr-1* gene in re-infections following administration of AL. Those polymorphisms were not associated with clinical treatment failure but are evidence for the ability of this drug combination to drive selection of parasites toward resistant phenotypes. Similarly, in Tanzania (Sisowath and others 2005) reported that Coartem selected for *pfmdr-1* polymorphisms associated with diminished sensitivity to lumefantrine. It is not clear if recrudescences after ACT were due primarily to inherent parasite resistance to artemisinins, resistance to the longer acting partner drugs, misclassification of some reinfections, or other factors. Therefore, associations between molecular markers, *in vitro drug* sensivity results, and clinical treatment outcomes need to be analyzed to detect early stages of resistance in this new era of antimalarial combination therapy.

Table 1 Summary of some Antimalarial combination clinicalefficacy studies in Africa

	Artemisinin combination therapy trail	Location of the trail	Number of patients in trail	Number of days follow up in clinical trail	Risk of reinfections %	Risk of recrudescences%	Year of publication
	Artemether + Lumefantrine	Kampala (Uganda)	105	28	6.7	1.0	*Dorsey et al.,2007*
	Artesunate + Amodiaquine	Kampala (Uganda)	113	28	17.4	4.6	*Dorsey et al.,2007*
	SP + Amodiaquine	Kampala (Uganda)	111	28	26.1	14	*Dorsey et al.,2007*
	Artemether + Lumefantrine	Burkina Faso	261	28	4.7	1.5%	*Zongo et al., 2007*
	SP + Amodiaquine	Burkina Faso	260	28	15.1	0.4	*Zongo et al., 2007*
1	Artesunate + Amodiaquine	Tororo (Uganda)	201	28	66%	0%	*Bukirwa et al., 2006*
2	Artemether + Lumefantrine	Tororo (Uganda)	202	28	51%	0.5%	*Bukirwa et al., 2006*
3	Artesunate + Amodiaquine	Four sentinal sites with different malarial intensity Uganda	705	28	14% - 68%	4% - 12%	*Yeka et al., 2005*
4	Artesunate + Amodiaquine	Tanzania	472	28	40%	11.2%	*Mutabingwa et al., 2005*
5	Artemether + Lumefantrine	Tanzania	485	28	21%	2.8%	*Mutabingwa et al., 2005*
6	Artesunate + Amodiaquine	Tanzania	181	42	36%	2.8%	*Martensson et al., 2005*
7	Artemether + Lumefantrine	Tanzania	182	42	17%	3.9%	*Martensson et al., 2005*
8	Artesunate + Amodiaquine	Pool province (Congo)	97	28	32%	1.5%	*Van den Broek et al., 2006*
9	Artemether + Lumefantrine	Pool province (Congo)	100	28	13%	0%	*Van den Broek et al., 2006*

10	Artesunate + Fansidar	Pool province (Congo)	85	28	25%	9.9%	*Van den Broek et al., 2006*
11	Artesunate + Amodiaquine	Kampala (Uganda)	103	28	12%		*Staedke et al., 2004*

2.14 In vitro antimalarial drug sensitivity assays

The *in vitro* drug senstivity assay is a laboratory tool to monitor trends of antimalarial drug susceptibility. As many countries resort to combination therapies to increase treatment efficacy and delay the emergence of drug-resistant parasites, monitoring the efficacy of individual components in drug combinations by *in vitro* drug senstivity assays and molecular markers is helpful (WHO.2005). The *in vitro* testing plays a role in detecting the early stages of resistance and has become a complementary tool for the surveillance of drug resistance. *In vitro* assays have the advantage of yielding objective results of parasite responses to drugs without any interference of host factors, including pharmacokinetics, acquired immunity, and patient compliance with therapy. The variations in parasite density and hematocrit (the inoculum effect), as well as the stage-dependent action of antimalarial drugs must be controlled because they cause a significant impact on the outcome of these assays(Duraisingh and others 1999; Zhang and others 1988). Parasites are cultured in erythrocytes in the presence of *RPMI 1640* media added to already dosed 96 well plates. Assays are run in duplicate for each drug, and placed in a candle jar at 37ºC for 72 hours. *In vitro drug*-sensitivity tests are all based on measurement of the effect of drugs on the growth and development of malaria parasites. These are parameters that can be measured in several different ways and each has exceptional characteristics. Parasite growth in drug-exposed cultures is measured relative to drug- free controls. When performed with serial dilution of drugs, these tests will result in sigmoid dose-response curves. Methods of *in vitro drug* sensitivity assays include the following: WHO microtest, isotopic (tritiated hypoxanthine uptake) assay, *lactate dehydrogenase (pLDH), histidine-rich protein 2(HRP2), SYBER Green 1* and other fluorescent dyes.

2.15 WHO microtest

Schizont maturation assays are relatively simple to perform (WHO, 1990), usually requiring only 24 hours of incubation. They require little technical equipment and can be used even for samples with low parasite densities. Blood samples are placed in already dosed 96 well plates. Assays are run in duplicate for each drug, and placed in a candle jar at 37ºC for 72 hours. Schizonts are counted against the total number of parasites on thick films prepared from the cellular layer of the cultured samples. Although this method is inexpensive, it is highly labor-intensive and subject to variations due to the expertise of the microscopists responsible for reading the slides. In addition, schizont maturation tests require highly trained personnel to limit individual variability in counting and assessing the developmental stages of the parasites.

2.16 Measuring parasitemia

Parasite growth may also be measured by assessing the increase in the percentage of infected red blood cells in the culture. Several drug-sensitivity assays were developed based on the measurement of the increase in parasitemia during a culture period of 48–96 hours(Nguyen-Dinh and others 1988; Richards and Maples 1979). These methods involve longer incubation times; they are applicable to most blood schizontocidal antimalarials, irrespective of the speed and stage specificity of their action. However, reading of the test results remains particularly labor intensive and, owing to the natural limitations of microscopy, is prone to variability of interpretation. Methods have therefore been developed that allow an automatic reading of drug-sensitivity tests in a flow cytometer(Saito-Ito and others 2001; van Vianen and others 1990), but these tests require highly sophisticated laboratory equipment.

2.17 Isotopic assay

The study done by (Desjardins and others 1979) developed an *in vitro* drug-sensitivity assay based on the incorporation of tritium-labeled hypoxanthine. As an alternative to using radiolabeled hypoxanthine, (Elabbadi and others 1992) proposed ethanolamine, which has the major advantage that the culture medium can be supplemented with hypoxanthine, resulting in improved parasite growth. Several other precursors (e.g. palmitate, serine, choline, inositol and isoleucine) have also been suggested for use in isotopic assays. These assays allow a fairly high degree of automatization and are therefore considerably faster to perform than are tests based on the morphological assessment of parasite growth. Automatic reading of the test results considerably reduces the influence of the variability caused by human factors. The assays are well suited for screening of antimalarial candidate compounds. Furthermore, they permit longer and variable incubation times, allowing the testing of a large variety of drugs. The fact that the measurement of the metabolic activity is limited to the second half of the culture period ensures a low and stable background. However, this technique also has its limitations, the foremost being its use of isotopic material. Since the late 1970s, the regulations regarding the handling of radioactive material have become considerably more restrictive, essentially aiming at limiting the application to domains where there is no alternative to their use. In many other fields, isotopic assays have therefore been replaced by other tests. Another limitation is the high purchase cost of the equipment, such as liquid scintillation counters and harvesting machines. In addition, the relatively high parasite densities of approximately 0.5% required for this test limits its application to the use of culture-adapted parasite strains or field samples with adequately high parasitemia.

2.18 Parasitelactate dehydrogenase

Parasite *lactate dehydrogenase* (*pLDH*) is a terminal enzyme in the *Embden–Meyerhof pathway (glycolysis)* of the malaria parasite (Makler and Hinrichs 1993). Its production and

accumulation can be used both in vivo and *in vitro as* indicators of parasite viability. *pLDH* was one of the first plasmodial enzymes shown to be electrophoretically, immunologically and kinetically distinct from that of the host(Sherman 1961). It was used primarily as an indicator for the presence of malaria parasites(Sherman 1961). The levels of *pLDH* correspond to the parasite density upon initial diagnosis (Makler and Hinrichs 1993). It was shown by(Sherman 1961) that a rapid decrease with the initiation of treatment with the resulting elimination of parasites (Oduola and others 1997). *pLDH* plays an important role in the anaerobic carbohydrate metabolism of human malaria parasites. As malaria parasites principally rely on anaerobic glycolysis, they require the regeneration of *nicotinamide adenine dinucleotide* (*NAD*) for the continuous flux of glucose through this pathway (Sherman 1979). On the basis of the knowledge that *pLDH* activity is distinguishable from host *LDH* activity using the *3-acetyl pyridine adenine dinucleotide* analog of *NAD* (*APAD*), (Makler and others 1993) developed a drug-sensitivity assay that determines inhibition profiles by measuring the enzymatic activity of *pLDH*. Pyruvate is formed from L-lactase in the presence of *LDH* and *APAD* coenzyme. This reaction results in the formation of reduced *APAD*, which in turn reduces blue tetrazolium, forming a blue formazan product that can be measured by spectrophotometry. However, the assay requires initial parasite densities of 1–2% and, in subsequent tests with fresh parasite isolates, was found to be too insensitive for field application (Basco and others 1995). The limitations associated with this test led to the development of a new *pLDH*-based assay that measures *pLDH* levels in a double-site enzyme-linked *LDH* immunodetection (*DELI*) assay that was made possible by the development of monoclonal antibodies (mAbs) specific for *pLDH*, making this test considerably more sensitive (Druilhe and others 2001). The *DELI* assay is equally applicable to the detection of parasites for diagnostic purposes as to drug-sensitivity testing. Studies from the same group suggest that, unlike its predecessor, the assay is also field applicable and

the results are comparable with those obtained by isotopic assay (Moreno and others 2001). However, limited supplies of *pLDH* monoclonal antibodies have constrained the further validation and application of the *DELI* assay.

2.19 Histidine-rich protein II

One of the most recent additions to the list of *in vitro* drug-sensitivity tests for *P. falciparum* is based on the measurement of a histidine- and alanine-rich protein produced by *P. falciparum* in the course of its growth and multiplication: *histidine-rich protein-2*(*HRP2*) (Noedl and others 2002). Similar to the *DELI* test, this assay is based on methods originally developed for the diagnosis of *P. falciparum* malaria (Wongsrichanalai and others 2001). It is 10 times more sensitive than the isotopic assay and requires little technical equipment. *HRP-2* levels are closely associated with parasite density and development(Desakorn and others 1997; Howard and others 1986). The *HRP-2* assay uses a longer culture time than most other assays (72 hrs. instead of 48 hours.), which also allows the testing of slow-acting drugs without changes in the protocol. Parasite growth and development is assessed by measuring the production of *HRP-2* in a simple, commercially available, double-site sandwich *ELISA* test kit (or essentially any *ELISA* that is specific to *HRP2*). The stability and persistence of *HRP-2*, which can be a major problem in predicting clinical treatment outcome using malaria rapid-diagnostic tests (dipsticks)(Mayxay and others 2001), turns into an advantage when using *HR-P2* for in vitro drug-sensitivity assays, as it provides a highly stable background. The background can be excluded by subtracting a control value taken after 24 hours from all results, thereby limiting the measurement of parasite growth to the later part of the culture. The data suggest that the results obtained with the *HRP-2* assay are comparable with those obtained by the *WHO* microtest and the isotopic assays. The assay works well with clones and laboratory strains of *P. falciparum* parasites, and with field isolates. It has the advantages of relative ease of implementation, the possibility of replacing isotopic tests, and reduced

requirements for laboratory equipment and training of personnel. As *ELISA*-based tests are easy and rapid to perform, even with large numbers of samples, they are also well suited for the screening of candidate drugs. Several other applications for these tests are also conceivable, such as bioassays or their use in vaccine development. The test has the limitation of requiring antibodies that are not widely available. In addition, in areas where genetic diversity is high, the use of monoclonal antibodies can be hindered by a decrease in binding affinities due to genetic variation within the protein (Baker and others 2005).

2.20 SYBR green I assay

Assessment of parasite growth can also be done by using *DNA* stains, which allows for a relatively simple reading of the results (Smeijsters and others 1996). It was reported by (Smilkstein and others 2004) independently that *SYBR green I* can be incorporated into the genomic *DNA* of malaria parasites for use as an *in vitro* assay. Parasite growth is determined by using *SYBR Green I*, a dye with marked fluorescence enhancement upon contact with *Plasmodium DNA*. The *SYBR green I* molecule intercalates in the genomic *DNA* of the parasite and fluoresces once it is in position in the *DNA*. The method yields results similar to those of the radioactive method at a fraction of the cost and without the production of harmful waste material (Corbett and others 2004). It has produced results comparable to other methods, showing that this method can be used routinely to conduct surveillance for drug resistance in *P. falciparum* with fresh or cultured parasites. It is simple, robust, inexpensive, one-step fluorescence assay for use in antimalarial drug screening (Bacon and others 2007). However, reading of fluorescence requires a spectrofluorometer, an expensive piece of equipment.

2.21 PicoGreen fluorescent dyes

Other fluorescent dyes have been used for *in vitro* drug senstivity assays(Corbett and others 2004; Smeijsters and others 1996). PicoGreen has been used and was found to produce results

comparable to those of the standard method based on the uptake of *[^{3}H]hypoxanthine* (Corbett and others 2004). A recently published study compared the *PicoGreen* method to isotopic and microscopic assays for the measurement of the CQ sensitivities of fresh and cryopreserved isolates of *P. vivax* (Kosaisavee and others 2006). The authors reported that there was no significant difference in the IC_{50} values, regardless of the method used. They also reported similar results with D6 (sensitive) and K (resistant) control isolates of *P. falciparum* (Kosaisavee and others 2006) but needs sophiscated expensive machines.

CHAPTER THREE

3.0 Aims

To distinguish known molecular markers associated with selection of resistance after ACT therapy, develop systems for studying fresh *Plasmodium falciparum* clinical isolates in Uganda, and assess associations between the *in vitro* drug sensitivity and genotypes of isolates and clinical outcomes after antimalarial therapy in Uganda.

3.1 Specific objectives

a) To assess the occurrence of various polymorphisms of a drug resistant allele of *pfmdr-1* and *pfcrt* halotypes between baseline and new infections during therapy with AS/AQ in Tororo Uganda.

b) To evaluate of changes in complexity of infection during short time culture of fresh clinical *Plasmodium falciparum* isolates in Kampala Uganda.

c) To assess the impact of various amodiaquine containing regimens on the *in vitro* sensitivity of recurrent *Plasmodium falciparum* isolates in Kampala Uganda.

d) To determine the *in vitro* senstivity patterns of *Plasmodium falciparum* fresh clinical isolates in Uganda against various antimalarial drugs in Kampala Uganda.

e) To evaluate associations between parasite genetic polymorphisms, *in vitro* drug sensitivity, and clinical outcomes after antimalarial therapy in Uganda.

3.2 Significance

The study will assist us monitor the sensitivity of AQ to *Plasmodium falciparum* in the population where it is being used in ACT therapy. In addition since the of mechanism of diminished AQ response is not fully explained, we will explore the use of known mutations in *pfmdr-1* and *pfcrt genes* to see if they can be used as molecular markers in monitoring AQ resistance in the population.

Complexity of infection in culture samples will assist us understand polyclonal infections how they behave as you subculture to see whether strains with mutations associated with resistance become less fit. *In vitro* senstivity of *P.falciparum* to antimalarial drugs will offer baseline IC_{50}'s, a valuable surveillance tool for monitoring antimalarial drug resistance in Uganda. Further, they will provide an early warning of impending resistance before it becomes clinically apparent. This study will also attempt to identify specific mutations that mediate resistance, and if such mutations are identified, their characterization will allow an improved understanding of mechanisms of antimalarial drug resistance and may allow the development of simple systems for the molecular surveillance of drug resistance in Uganda. A thorough understanding of how mutations in genes encoding putative transport proteins influence parasite response to antimalarial drugs will be helpful in worldwide efforts to control this important disease.

CHAPTER FOUR

MATERIALS AND METHODS

Various study designs were used to address the different objectives:

4.1 Study design 1

To address the question as to whether changes in occurrence of various polymorphisms of a drug resistant allele *pfmdr-1* and *pfcrt* halotypes differ between baseline and new infections during therapy with different treatment arms, the study utilized samples from a clinical trial comparing the efficacies of AL and AQ/AS in Tororo, Uganda, a region of very high malaria transmission (Bukirwa and others 2006). Children aged 1 to 10 years with uncomplicated malaria were randomized to receive directly observed therapy with one of the ACTs and followed for 28 days. Treatment responses were monitored based on WHO criteria. For episodes of recurrent parasitemia that occurred more than 3 days after therapy, polymorphisms in merozoite surface protein-2 (*msp*-2) were compared to distinguish recrudescent from new infections, as previously described (Cattamanchi and others 2003).Outcomes were classified as recrudescence if all *msp-2* alleles present on the day of failure were present on the day of enrollment and new infections if new alleles had arisen. The clinical trial was approved by the Uganda National Council of Science and Technology and the Institutional Review Boards of Makerere University, the University of California, San Francisco, and the University of California, Berkeley.

We evaluated polymorphisms at the *pfmdr-1* alleles *N86Y, Y184F, S1034C, N1042D,* and *D1246Y* in 201 isolates collected before the initiation of treatment and 132 isolates from new infections in AQ/AS arm that presented over 28 days of follow-up after therapy. Blood was collected on filter paper on the day of initial diagnosis and at the time of new infection. *DNA* was isolated by Chelex extraction (Plowe and Wellems 1995). Briefly, blood spots of appropriate size were cut, soaked, inverted several times and stored for 4 hours at 4^{o}C in a

tube containing 1mL of phosphate-buffered saline (*PBS*) and 50uL of 10% saponin at 4°C. The microfuge tube was centrifuged at 13,000 rpm for 5 seconds and the *PBS*/saponin aspirated. After discarding the supernatant, 1mL of *PBS* (no saponin) was added, the tube was inverted several times and it was then incubated at 4^{o}C for 30 minutes. The microfuge tube was centrifuged at 13,000 rpm for two minutes to wash the sediment. After discarding the supernatant, 100µL of sterile water was added, and 50µL of vortexed chelex stock solution was dispensed to each tube. Parasite *DNA* was extracted by incubating tubes for 10 minutes in a 95°C heat block coupled with vigorous vortexing of each sample after two minutes during the incubation. After incubation tubes were microfuged for 5 minutes at high speed and 200uL was transfered into a tube. The tube was then microfuged for 10 minutes and the final white to yellow supernatant was transferred to another labeled tube. To analyse polymorphisms at codons *86, 184, 1034, 1042, and 1246* of the *pfmdr-1* gene, flanking sequences were amplified by *PCR* and digested with specific restriction enzymes as described by(Duraisingh and others 2000a)see the table 2.

Table 2. PCR primers for *pfmdr-1* for single nucleotide polymorphisms

Pfmdr-1 *86 & 184 1st round*	*MDR-A1:(30bp) (5'-TGT TGA AAG ATG GGTA AAG AGC AGA AAG AG-3')* *MDR-A3:(33bp) (5'TAC TTT CTT ATT ACA TAT GAC ACC ACA AAC A-3')*
Pfmdr-1 *86 & 184 2nd round*	*MDR-A4: (30bp) (5'-AAA GAT GGT AAC CTC AGT ATC AAA GAA GAG -3')* *MDR-A2:(33bp) (5'- GTC AAA CGT GCA TTT TTT ATT AAT GAC CAT TTA-3')*
Pfmdr-1 1034, 1042&1246 *1st round*	*MDR-01 :(33bp) (5'-AGA AGA TTA TTT CTG TAA TTT GAT ACA AAA AGC-3')* *MDR-02:(30bp) (5'-ATG ATT CGA TAA ATT CAT CTA TAG CAG CAA-3')*
Pfmdr-1 1034&1042 2nd round	*1034F :(33bp) (5'-AGA ATT ATT GTA AAT GCA GCT TTA TGG GGA CTC-3')* *1042R:(33bp) (5'-AAT GGA TAA TAT TTC TCA AAT GAT AAC TTA GCA-3')*
Pfmdr-1 1246 2nd round	*1246F:(35bp) (5'-ATG ATC ACA TTA TAT TAA AAA ATG ATA TGA CAA AT-3')* *MDR-02 :(30bp) (5'-ATG ATT CGA TAA ATT CAT CTA TAG CAG CAA-3')*

From M.T Duraisingh et al. Mol Biochem Parasitol.2000 Apr 30; 108(1:13-23)

The reaction conditions for both the primary and nested PCR were as published previously (Duraisingh and others 1998). The restriction enzymes: *ApoI, DraI, DdeI, VspI and EcoRV* were used to detect polymorphisms at codons *86, 184, 1034, 1042,* and *1246* of the *pfmdr-1* gene, respectively. The genomic *DNA* of the *K1, W2* and *7G8* laboratory isolates was used as positive controls for wild type and mutant, doubly distilled water as negative control. Controls were concurrently amplified, digested and run alongside the experimental samples. Restriction digestion was carried out according to manufacturer's instructions (New England Biolabs).

Alleles were identified using nested *PCR* and restriction fragment length polymorphism methods, as previously described (Duraisingh and others 2000a). Digestion products were resolved by gel electrophoresis, and results classified as wild type, mutant, or mixed based on migration patterns of *ethidium bromide* stained fragments.

Polymorphisms at codons *72, 74, 75* and *76* in the *pfcrt* gene were determined in 90 randomly selected pretreatment and 90 randomly selected new infection isolates using a *PCR-allele specific restriction analysis* method as described (Djimde and others 2001). The sequence flanking codons 72, 74, 75 and 76 was amplified as shown in the table 3 below:

pfcrt72,74,75, 76 1st round	*76-A: (5'GCG CGCGCATGGCTCACGTTTAGGTGGAG3')* *76-B: (5' GGGCCCGGCGGATGTTACAAAACTATAGTTACC3')*
Pfcrt 76 2nd round	*76D1: (5'TGTGCTCATGTGTTTAAACTT3')* *76-D2: (5'CAAAACTATAGTTACCAATTTTG3').*
Pfcrt 72 2nd round	*CRT72MS: (5'TTTATATTTTAAGTA TTATTTATTTAA GTG GA3')* *76-D2: (5'CAAAACTATAGTTACCAATTTTG3').*
Pfcrt 74, 75 2nd round	*CRT745MS: (5'TAA GTATTATTTATTTAAGTGTATGTGTCAT3')* *76-D2: (5'CAAAACTATAGTTACCAATTTTG3').*

Table 3 PCR primers for *pfcrt halotypes*.for single nucleotide polymorphisms
From http://medschool.umaryland.edu/cvd/2002_pcr_asra.asp

The reaction conditions for both the primary and nested PCR were as published previously(Djimde and others 2001). The restriction enzymes *Fok1, NIaIII, BspHI, ApoI* were used to detect SNP's for 72,74,75,76 respectively.

The genomic *DNA of HB3, DIV, Dd2* and *7G8* laboratory isolates were used as positive controls and doubly distilled water as a negative control. Controls were amplified, digested and run alongside the samples. Restriction digestion was carried out according to manufacturer's instructions (New England Biolabs). Restriction digestion products was examined by electrophoresis on 2.5% agarose gels.

Data Analysis

Data were entered and verified using SPSS and analyzed using STATA 8.0. The prevalences of mutations in pretreatment and new infection isolates were compared using the chi-squared or Fisher's exact test as appropriate. A p-value <0.05 was considered statistically significant.

4.2 Study design 2

To evaluate changes in complexity of infection during short time culture of fresh clinical *Plasmodium falciparum* isolates**,** samples were collected from a cohort of children in the Mulago III Parish of Kampala enrolled in a longitudinal drug efficacy comparison by (Dorsey and others 2007). The clinical trial began in November, 2004; samples for this study were collected between October, 2005 and May, 2006. The clinical trial and analysis of cultured parasites were approved by the Uganda National Council of Science and Technology, the Makerere University Research and Ethics Committee, and the University of California, San Francisco Committee on Human Research.

At the time of new diagnosis of malaria blood was spotted onto filter paper (Whatman 3MM), collected in an EDTA tube, and transported within 30 minutes to our laboratory. Giemsa-stained thin blood smears were examined, and if *P. falciparum* infection and the lack of other plasmodial species were confirmed, culture was initiated. Blood was centrifuged, plasma and

buffy coat were removed, and the erythrocyte pellet was washed with RPMI 1640 medium supplemented with 25 mM HEPES, 0.2% $NaHCO_3$, 0.1 mM hypoxanthine, and 0.25% Albumax II serum substitute. Washed erythrocyte pellets (200 µl) were used to inoculate cultures in the same medium at 2% hematocrit in 10 ml sterile flasks. Parasites were then placed in a candle jar or at 5% CO_2 at 37ºC. Every 24 h, Giemsa-stained smears of cultures were examined, blood was spotted onto filter paper, and culture medium (prepared fresh at least every 2 weeks) was replaced. All cultures in which parasites remained for 9 days were considered in this analysis. For analysis of COI, parasite DNA was extracted from filter paper using Chelex100 resin, and a variant-specific nested PCR protocol was used to amplify either the *3D7* or *FC27* variants of the *msp-2* gene, as previously described(Cattamanchi and others 2003). PCR products were resolved on agarose gels, and the size of products was compared to standards and between days on densitometric digitized gel images analyzed by GelCompar II software (Applied Maths). Strains from different days were considered identical if estimated fragment lengths were within 15 base pairs.

For samples which had mixed infection at day 0 and lost some strains within days, polymorphisms in individual loci of three genes, *dhfr C59R*, *dhps K540E*, and *pfmdr-1 N86Y*, were determined by nested PCR of relevant portions of genes followed by sequence-specific restriction endonuclease digestion (Duraisingh and others 1998) and (Duraisingh and others 2000a) resolution of digestion products by agarose gel electrophoresis, and analysis of densitometric digitized gel images using GelCompare II.

Statistical analysis

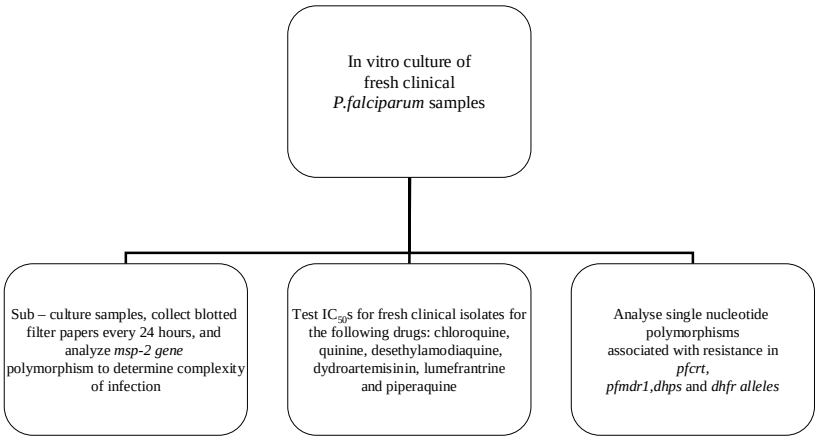
We used a one sample test of proportions to test the null hypothesis that equal proportions of mutant and wild type parasites would be selected in culture.

4.3 Study design 3

To determine *in vitro* senstivity patterns of *P. falciparum* fresh clinical isolates in Uganda against various antimalarial drugs, blood samples for *in vitro* sensitivity were collected from the same study as for study design 2. Briefly,the cohort study was a randomized longitudinal clinical trail comparing the efficacies of different combination antimalarial regimens for the treatment of uncomplicated malaria in Uganda children (Dorsey and others 2007). The study design diagram is shown in figure 1.

Figure 1

Figure 1 Confirmed *Plasmodium falciparum* fresh clinical isolates flow chart analysis in the laboratory



In vitro drug sensitivity was assessed on fresh clinical isolates using (Noedl and others 2004) (Noedl and others 2005) established protocols figure 1. Briefly, 96 well plates were set up with serial dilutions of different antimalarials, which allowed construction of dose response curves. In brief, multiple wells of a 96-well culture plate were pre-dosed with 2-fold serial dilutions of each drug of interest, with concentrations based on clinically relevant ranges for each drug. Plates were set up immediately before assessments of drug sensitivity. Wells were dried in an incubator and stored at 4ºC in sealed plastic bags. We used 200 ul of blood pellet from each fresh clinical sample and added it to 10 ml *RPMI 1640* media contained in a conical tube to produce a packed cell volume of approximately 1.5%. Higher parasite densities were diluted with 1.5% blood group O uninfected RBC to obtain a density of 0.05%, to avoid an inoculum effect. 200 ul of 1.5% packed cell volume of uninfected red cells and 0.05% infected red blood cells were added in each already dosed 96 well plate with each concentration run in duplicate for each drug(table 3), and placed in a candle jar at 37ºC for 72 hours. After the incubation, a blood smear was prepared to confirm healthy growth of controls and determine the percentage of infected red blood cells on a thin smear to establish the rate of growth.

Table 4 antimalarial drugs analyzed

Antimalarial drugs	Cat No.	Vendor
Quinine Sulfate	Q0132-10g	Sigma-Aldrich
Piparaquine	Cas 4035-33-8	Porton International Ltd
Chloquine Diphosphate crystalline	C6628-50g	Sigma-Aldrich
Desethylamodiaquine	451782	BD Gentest
Lumefantrine	Batch. 05082801	Dafra PHARM
Dihydroartemisinin	030115- 206DHA	Dafra PHARM

HRP-2 ELISA assay

Drug sensitivity was assessed using an *ELISA assay* that quantifies parasites based on expression of *histidine rich protein-2 (HRP-2),* as optimized by (Noedl and others 2004). Briefly, the IgM capture antibody (*MPFM-45A*) specific for *P. falciparum HRP-2* was diluted to 1ug/ml in PBS and 100ul transferred/well to a 96-well plate. Plates were covered and placed at 4^0 C overnight. After incubation, the content was removed and plates were washed three times with *PBS*. The non- specific sites were blocked using 200ul/well of 2% *bovine serum albumin* diluted in *PBS.* The plates were washed as before and 100 ul of the hemolysate from parasites grown for 72 hours in the absence or presence of antimalarial drugs were added and incubated for one hour at room temperature. Plates were washed again three times and 100 ul of the detector antibody conjugated to *hoseradish peroxidase (MPFG-45)* was diluted to 0.05g/ml in *PBS,* added to each well and incubated at 37ºC. A subsequent washing followed, and 100 ul of *nitrotetrazolium blue chromagen* was added per well and incubated for 10 minutes, followed by adding 50 ul of 1M sulphuric acid to stop the reaction. Absorbance was determined using a *VERSAmax spectraphotometer* at a wavelength of 450nm. *ELISA* results for experimental and control cultures were used to construct a dose-response curve for each drug, and IC_{50}'s calculated using *HN – Non – Linear regression softwareVI.I,* with data fitted by nonlinear regression to a variable-slope sigmoidal dose-response formula (Noedl and others 2002).

Statistical analysis

Inhibitory concentrations (IC_{50}s) for antimalarials was calculated using *HN-nonlin VI.05 beta software* (Noedl and others 2005). The software used is based on a polynomial regression model and is freely available from http; // malaria. Farch. net. The *in vitro activity* of antimalarials was expressed as the geometric mean of the IC_{50}s from all isolates. To measure the possible differences in activity, the mean logarithmic IC_{50} values of the antimalarials were compared by the two-tailed unpaired Student t test. Potential *in vitro* cross – resistance was

evaluated by Pearson correlation. For all statistical tests, the significance level (p value) was set at 0.05. Comparison of mean IC_{50}'s for 2007 and 2008 were estimated by the non – parametric Mann Whitney test.

Evaluation of specific mutations

We calculated frequencies of mutant genotypes in two groups stratified into most and least sensitivity IC_{50}s for the same drug. The identified molecular markers from *in vitro studies* were associated with clinical treatment outcomes. We assessed associations between individual mutations using chi- square test or fisher's exact test as appropriate. We assessed specific mutations alone and in combination and treatment failure using generalized estimating equations and the *STATA* statistical program for data analyses (State, College Station, Texas).

4.4 Study design 4

To assess the impact of various amodiaquine containing regimens on the sensitivity of recurrent *P. falciparum* isolates in Uganda. Still a subset of samples were from a cohort of 601 children randomly selected from a community in Kampala, Uganda, aged 1-10 years at enrollment in 2004-05, as previously described (Dorsey and others 2007). Upon presentation with the first episode of uncomplicated malaria, participants were randomly assigned to receive AQ/SP, AS/AQ, or AL, which they received thereafter for each episode of uncomplicated malaria. With each treatment, efficacy was assessed following WHO guidelines WHO2003 , with genotyping to distinguish recrudescence and new infection after therapy, all as previously described(Cattamanchi and others 2003). We assessed the selective pressure for resistance of AQ, which was a component of two of our study regimens, AQ/SP and AS/AQ. For the 61 samples for which IC_{50} data were available, we compared the sensitivity of parasites collected from subjects who experienced a new infection within 12 weeks of a prior infection treated with AQ with those from subjects who had not had recent

prior therapy with AQ. We additionally compared the sensitivity of parasites from subjects who experienced a new infection within 12 weeks of a prior treatment with AQ with only parasites from the non-AQ (AL) treatment arm (thus subjects who never received AQ). We evaluated the *in vitro* activity of the primary active metabolite of AQ, monodesethylamodiaquine (MDAQ), against 61 fresh clinical samples collected from Ugandan children before treatment for uncomplicated falciparum malaria with one of three combination regimens, two of which included AQ. Methods for IC_{50} determination were exactly as described in study design3 above. Breifly upon diagnosis of uncomplicated malaria, and before the initiation of therapy, blood was collected into heparinized tubes and delivered promptly to our laboratory. Specimens were centrifuged, supernatant and buffy coat were removed, erythrocytes were washed twice in RPMI 1640 medium pre-warmed to 37°C, samples were diluted to 0.05% parasitemia and 2% hematocrit, and parasites were cultured under sterile conditions in RPMI 1640 supplemented with 0.5% Albumax and 100 µg/ml gentamicin. To test *in vitro drug* sensitivity, 96-well cell culture plates were coated with 7 serial dilutions of MDAQ (6.25-400 nM) and dried overnight. Wells without drug served as controls. For each sample, 200 µl of culture was added to each well, with duplicate wells for each concentration of MDAQ. Plates were incubated for 72 hours at 37°C in a candle jar, and samples were then frozen (-20°C overnight) and thawed before analysis. Parasite growth was assayed by comparing levels of *histidine rich protein-2 (HRP-2)* in treated and control cultures. *HRP-2* was quantified using a commercial *ELISA* test kit (Malaria Ag Celisa, Cellabs). Samples were diluted (1:4-1:10; the same dilution for each sample in an experiment) in water, and 100 µl of each hemolyzed sample preparation was added to an *ELISA* plate pre-coated with *anti-HRP-2* and incubated at room temperature for 1 hour. Plates were then washed four times with the kit washing solution, 100 µl of secondary antibody was added to each well for1 h at room temperature, plates were again washed 4 times, 100 µl of

chromogen substrate was added to each well, plates were incubated for 15 min in the dark, and 50 µl of stopping solution was added. Absorbance (450 nm) was then measured for each well with a SpectraMAX 340 spectrophotometer (Molecular Devices). Optical density values were fitted to normal curves based on serial dilutions of *HRP-2* standards and a four-parameter curve model (Softmax Pro 2.1.1, Molecular Devices), and IC_{50}s were calculated based on a nonlinear regression model (http://www.meduniwien.ac.at/user/harald.noedl/malaria/).

Statistical analyses

Clinical data were entered and verified with Access (Microsoft Corporation). Statistical analyses were performed with Stata, version 10 (StataCorp). Clinical outcomes were assessed as previously described(Dorsey and others 2007). Values for *in vitro* drug sensitivity of parasite samples were not normally distributed, and were assessed using the Kruskal-Wallis rank test. Prevalences of mutations were compared with Fisher's exact test. For all assessments, p values <0.05 were considered statistically significant.

CHAPTER FIVE

RESULTS

5.1 Selection of plasmodium falciparum resistance-mediating pfcrt and pfmdr-1 alleles after treatment with artesunate-amodiaquine in Tororo Uganda

The selection of resistant parasites, particularly to artemisinin partner drugs, remains a concern in Africa. In order to assess changes in occurrence of various polymorphisms of a drug resistant alleles of *pfmdr-1 and pfcrt* between baseline and new infections during AS/AQ therapy, our clinical trial identified no recrudescences, but frequent new infections (66% of subjects) within a month after treatment of uncomplicated malaria. We evaluated polymorphisms at the *pfmdr-1* alleles (*N86Y, Y184F, S1034C, N1042D, D1246Y*) in 201 isolates collected before the initiation of treatment representing all patients randomized to therapy with (AS/AQ) and 132 isolates from all new infections that presented over 28 days of follow-up after therapy. We also evaluated *pfcrt* haplotypes at positions 72-76 in 90 randomly selected pretreatment and 90 randomly selected new infection isolates. Alleles were identified using nested PCR and restriction fragment length polymorphism methods. Genotyping was successful for all isolates and *pfmdr-1* prevalence of mutant alleles (including mixed and pure mutant isolates) increased significantly from pretreatment to new infection isolates for *86Y* (182/201, 90.5% to 128/132, 97.0%, p=0.03), *1246Y* (167/201, 83.1% to 120/132, 90.9%, p=0.04), and these two mutations together (164/201, 81.6% to 119/132, 90.2%, p=0.03; Figure 2. In contrast, the prevalence of the wild type allele Y184 increased from pretreatment to new infection isolates (171/201, 85.1% to 122/132, 92.4%, p=0.04). Only wild type alleles were seen at positions 1034 and 1042 in both pretreatments and new infections. For *pfcrt*, the *CVIET* haplotype at positions *72-76* was seen in all 180 samples analyzed.

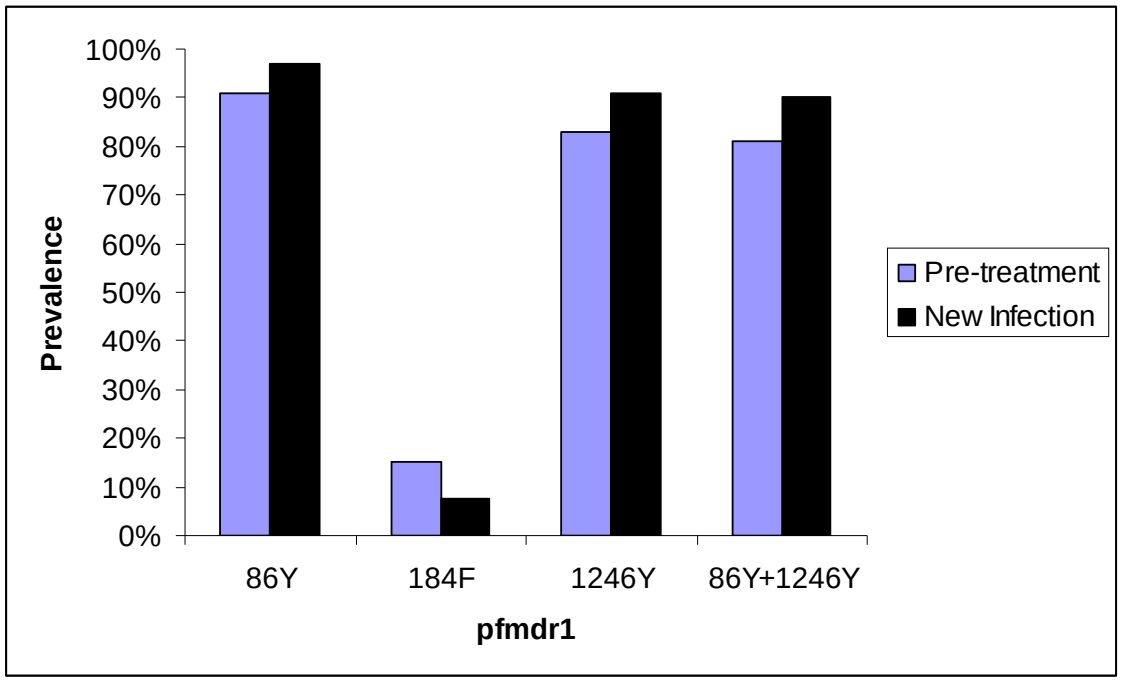


Figure 2 Selection of *pfmdr-1* alleles after therapy with AS/AQ.

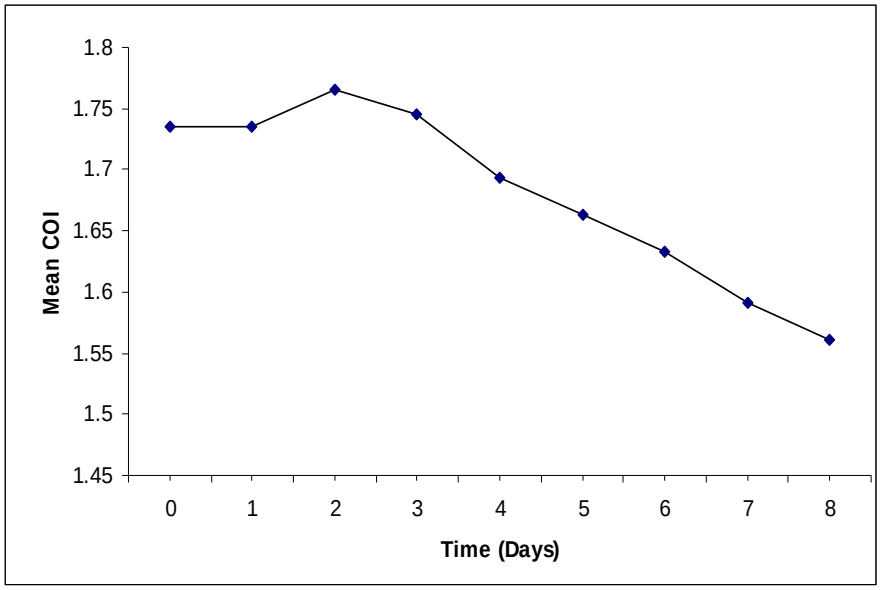
The proportion of isolates that were mutant at the indicated alleles is shown. Differences between genotypes in pre-treatment and new infection isolates were statistically significant in all cases (p0<.05) using chi square.

5.2 Complexity of plasmodium faciparum clinical samples during short-term culture

Culture of fresh parasites is widely used to study the *in vitro* drug sensitivity of infecting parasites and to search for associations between parasite features and clinical outcomes. However *P. falciparum* infections are commonly polyclonal in many areas, and, since not all parasites successfully grow in culture, it is unclear how well *in vitro* culture represents the complexity of clinical infections. To characterize changes in COI during short-term culture of *P. falciparum*, samples were collected from a cohort of children in the Mulago III Parish of Kampala enrolled in a longitudinal drug efficacy comparison (Dorsey and others 2007). The clinical trial began in November, 2004; samples for this study were collected between October, 2005 and May, 2006. A total of 211 samples were cultured from children in Kampala (aged 1 – 10 years at the time of study enrollment) with acute malaria. Complexity of infection was measured by assessment of the diversity of highly polymorphic *P. falciparum* genes, most commonly that encoding merozoite surface protein-2 (*msp-2*). Of the samples collected, 98(46%) were successfully cultured for at least 9 days. Other samples were not maintained due to failure of parasites to survive for 9 days (100 cultures) or bacterial contamination (13 cultures). Parasite density at diagnosis was greater in samples that successfully grew for 9 days (mean 30,400 parasites/µl) than in those that did not (mean 11,500/µl). COI was assessed daily based on analysis of polymorphic regions of *msp-2*. Of the 98 samples that grew, for 53(54%) a single genotype was detected at the time of initiation of culture (Day 0). The mean COI on day 0 was 1.73 (95% CI 1.56 - 1.92); the COI was stable for the first 4 days of culture, and then decreased gradually to 1.56 (95% CI 1.40-1.73) on day 8 (figure 3A). To gain insight into the sensitivity of single time point assessment of *COI*, we followed the appearance of new strains after day 0. Strains not present on Day 0 were first identified on days 1-5; 26 new strains in 20 cultures were identified (figure 3B) .We were also interested in the loss of strains after day 0. In 25(56%) of the 45 cultures that

were polyclonal on Day 0, some strains disappeared over 9 days of culture (figure 3A&B). The total number of strains that disappeared was 32. We examined the possibility that parasite fitness, considered here as the ability of a parasite to survive in culture, played a role in the disappearance of certain strains. Fitness might be diminished in drug-resistant parasites and wild type parasites appears after drug pressure We therefore evaluated three resistance-mediating polymorphisms in mixed cultures that subsequently became monoclonal (within our limits of detection): the key mediators of antifolate resistance in East Africa, *dhfr C59R* and *dhps K540E* and a mediator of responses to a number of antimalarials, *pfmdr-1 N86Y* determined by nested PCR of relevant portions of genes followed by sequence-specific restriction endonuclease digestion. For 12 cultures that had mixed genotypes at *dhfr* 59 on day 0, 5 subsequently were monoclonal, 4 *C59* and 1 *59R*, on day 8. For 21 cultures that were mixed at *dhps* 540 on day 0, 2 subsequently were monoclonal, both pure wild type *K540*, on day 8. Thus, for the mutations that mediate SP resistance, when cultures became monoclonal, wild type parasites persisted in 6 of 7 instances. With the small available sample size, results did not differ significantly from a random distribution of alleles (p=0.18 and 0.16 for selection of *C59* and *K540*, respectively). For 19 cultures that were mixed at *pfmdr-1 86* on day 0, 5 subsequently were monoclonal, all with the *86Y* genotype, on day 8, a significant selection for *86Y* in culture (p=0.03).

A



B

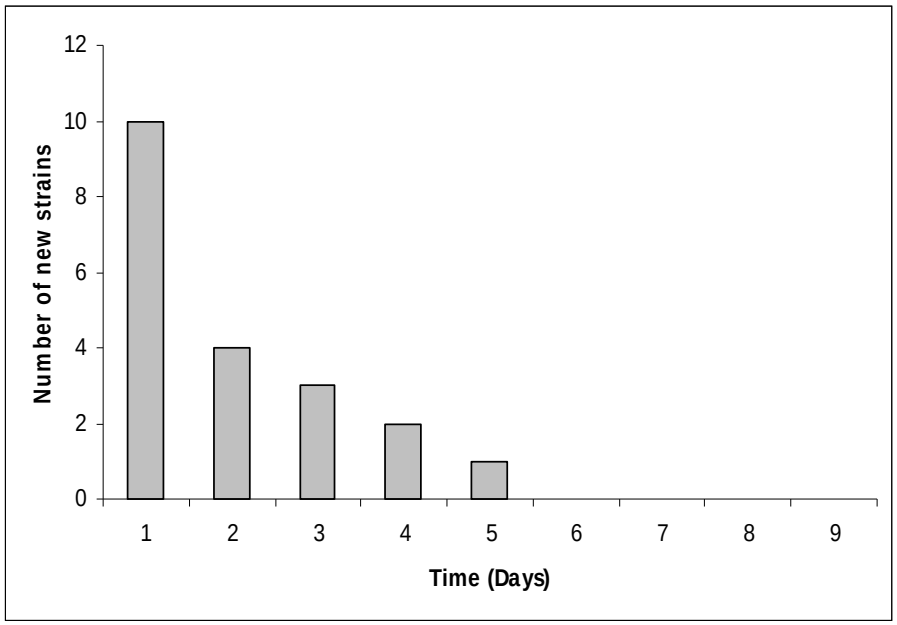


Figure 3 COI during short term culture.

A. Mean Complexity of infection (COI) in culture over time. Cultures were initiated on day 0, and COI was determined daily. **B.** Number of strains identified for the first time after day 0. The day on which strains were first identified is shown.

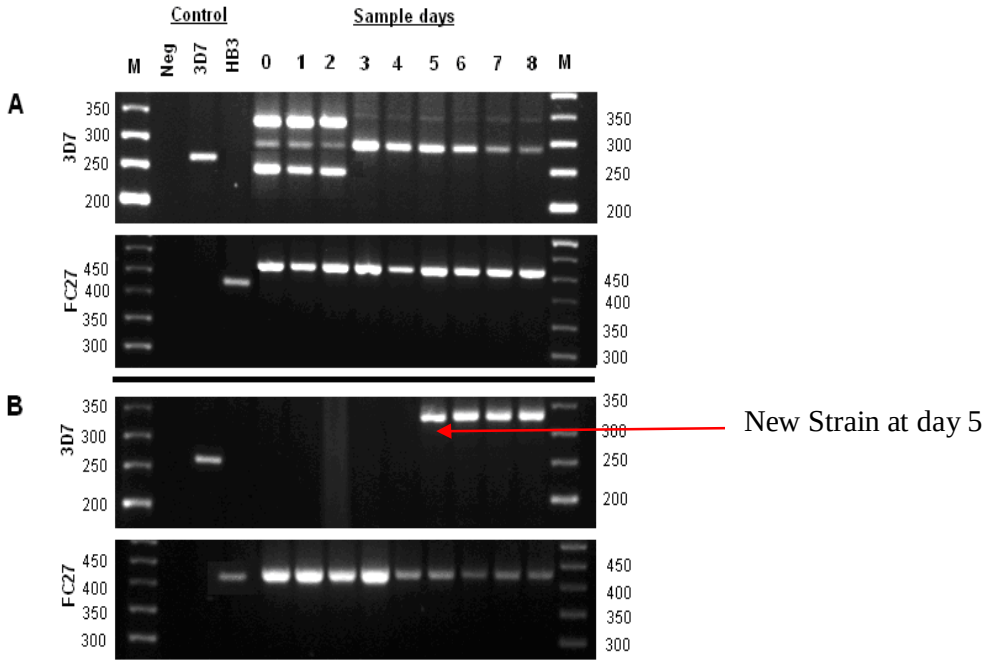


Figure 4 Genotype patterns over time for two representative cultures.
For each culture, *DNA* was extracted daily, the *3D7* and *FC27* alleles of *msp-2* were amplified, and products were resolved by agarose gel electrophoresis. Controls included no *DNA* (Neg) and *DNA* from laboratory strains known to express the *3D7 (3D7)* or *FC27 (HB3*) allele. Sizes were compared based on 50 bp markers (M). The results shown demonstrate a culture with initial COI of 4, with loss of 2 *3D7* strains after day 2 (A) and a culture with initial COI of 1, with appearance of an additional 3D7 strain on day 5 (B).

5.3 Selection of parasites with diminished drug sensitivity by amodiaquine-containing antimalarial regimens in Uganda

As we move to routine treatment of malaria with combination regimens, it is unclear how readily resistance to AQ will be selected by treatment with AQ-containing combinations. Amodiaquine (AQ) is paired with artesunate (AS) or sulfadoxine-pyrimethamine (SP) in recommended antimalarial regimens. To gain insight of various amodiaquine containing regimens on the sensitivity of recurrent *P. falciparum* isolates in Uganda, we collected 61 *Plasmodium falciparum* samples from a cohort of Ugandan children randomized to treatment with AQ/SP, AS/AQ, or artemether-lumefantrine (AL) for uncomplicated malaria. Upon presentation with the first episode of uncomplicated malaria, participants were randomly assigned to receive AQ/SP, AS/AQ, or AL, which they received thereafter for each episode of uncomplicated malaria. With each treatment, efficacy was assessed following WHO guidelines , with genotyping to distinguish recrudescence and new infection after therapy, all as previously described by (Cattamanchi and others 2003). For this study we analyzed a subset of samples, collected between August, 2006 and March, 2007 for which parasites were successfully grown in short-term culture to allow determination of *in vitro drug* sensitivity. *In vitro sensitivity* to monodesethylamodiaquine was measured with a *histidine rich protein-2-based ELISA* and potential resistance-mediating polymorphisms in *pfmdr-1* were evaluated by nested PCR followed by mutation-specific restriction endonuclease. We then searched for associations between *in vitro drug* sensitivity, clinical outcomes, prior drug use, and known molecular mediators of resistance. Most notably, we found that prior use of AQ-containing combinations selected for subsequent infections with diminished responsiveness to MDAQ.

Among 72 samples cultured 1 was contaminated, 4 died, 6 did not have a good fit log dose curve, and 61 were successful with a wide range of *in vitro drug* sensitivity as seen in (figure 5). Using a standard cut-off for resistance of IC_{50} >60 nM , 13 (21.3%) of the parasite samples were categorized as resistant to MDAQ, and using a more rigorous cut-off of 100 nM, 8 (13.1%) were highly resistant.

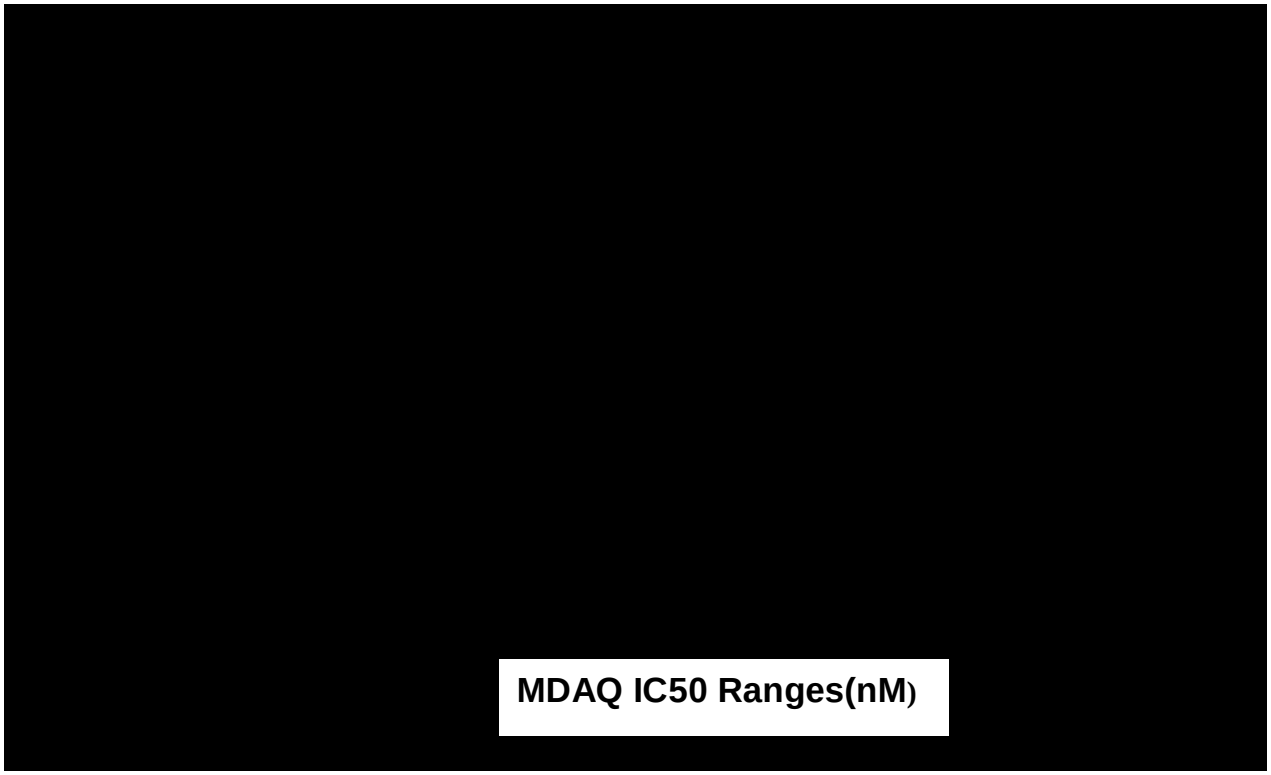


Figure 5 ***In vitro drug*** **sensitivity using monodesethylamodiaquine(MDAQ)**
Fresh *P.falciparum* clinical isolateswere assessed for susceptibility to MDAQ by comparing levels of HRP-2 with those of controls after incubation with multiple concentrations of MDAQ. OD values from *ELISA* assays were fitted to normal curves and transformed into HRP-2 values using a four-parameter curve model (Softmax Pro 2.1.1, Molecular Devices). A nonlinear regression model *(HN-nonlin VI.05 beta software*; (Noedl and others 2005)was used to calculate 50% inhibitory concentrations (IC_{50}s). IC_{50} values for the 61 studied samples are shown.

5.3.1 Association of *in vitro* drug sensitivity and clinical outcomes

The three combination regimens included in our clinical trial were all quite efficacious, and so few recrudescences occurred after therapy . Only 7 of the 61 samples (11.5%) were from episodes of malaria classified as recrudescent. For these samples, and for both the AQ-containing treatment arms and the AL arm, recrudescence was equally likely in those with parasites sensitive and resistant to MDAQ (table 5). Thus, although sample size was small for this analysis, we saw no association between MDAQ sensitivity and treatment outcome.

Table 5 Association between *in vitro* MDAQ sensitivity and clinical outcomes
No association was seen between *in vitro* sensitivity to MDAQ and outcome after treatment with three different combination regimens. Treatment regimens were AQ/SP, AQ/AS, and AL.

Study regimen	**MDAQ sensitivity (IC_{50}, nM)**	**Number of strains**	**Number of strains with recrudescent outcomes (%)**
AQ/SP	< 60	10	2 (20)
	> 60	5	1 (20)
AQ/AS	< 60	18	1 (5.6)
	> 60	6	0
AL	< 60	20	2 (10)
	> 60	2	1 (50)

5.3.2 Parasites with diminished sensitivity to MDAQ were selected by prior therapy with AQ

To assess the impact on drug sensitivity of recent prior therapy, we compared the MDAQ sensitivity of parasites from subjects that were previously treated with AQ/SP or AS/AQ within 12 weeks with those from patients not treated with these drugs within this interval and with only those from patients in the AL treatment arm, and so not treated with AQ during the course of the study. Parasites from subjects who were previously treated with AQ/SP or AS/AQ within 12 weeks were less sensitive to AQ (n=18; mean IC_{50} 62.9 nM; range 12.7-158.3 nM) compared to parasites from those not treated with an AQ-containing regimen within 12 weeks (n=43; mean IC_{50} 37.5 nM; p=0.0085; range 6.3-184.7 nM) or compared to parasites from those in the treatment arm that did not contain AQ (AL subjects; n=20; mean IC_{50} 28.8 nM; p=0.0042; range 6.3-121.8 nM) (figure 6). Similar associations were seen when drug sensitivities were compared only for parasites causing new infections and for infections occurring within 6, 8, or 10 weeks of prior therapy, although with intervals $\leq$6 weeks differences in drug sensitivities were not statistically significant

Figure 6 Selection of parasites with diminished response to amodiaquine.
In vitro sensitivities were determined and results are plotted for parasites from all patients that did not receive an amodaquine-containing regimen within 12 weeks (No Prior amodaquine; A) or only patients in the artesunate/lumefrantrine(AL) treatment arm, who did not receive an amodiaquine-containing regimen for the entire course of the study (No Prior amodiaquine; B), in both cases compared to results for subjects who did receive an amodiaquine-containing regimen within 12 weeks prior to the time of this analysis (Prior amodiaquine). Means are indicated by horizontal lines. Differences for both comparisons were significant *p value 0.05*

Table 6 *In vitro* sensitivity of monodesethylamodiaquine(MDAQ) to parasites causing infections soon after prior treatment with artesunate/amodiaquine therapy with time.

Time since prior treatment with AQ	Average MDAQ IC_{50}, nM	n	p value[1]
6 weeks	46.4	11	0.076
8 weeks	54.5	14	0.019
10 weeks	57.3	17	0.0073
12 weeks	62.9	18	0.0042

[1]Mean IC_{50} values were compared with those for samples from patients in the AL treatment arm (n=20; mean IC_{50} 28.8 nM) by the Kruskal Wallis test.

5.3.3 Selection of parasite genetic polymorphisms by prior therapy with AQ

We also characterized genetic polymorphisms that have previously been identified in the *pfmdr-1* genes seen previously (Basco and Ringwald 2007) (Dokomajilar and others 2006a) (Dorsey and others 2007) (Happi and others 2006) prior treatment with AQ (within 12 weeks) selected for two *pfmdr-1* polymorphisms, *86Y and 1246Y*, that have been associated with decreased responsiveness to this drug, although baseline prevalences of these polymorphisms were high, and differences were not statistically significant(Chi Square Test)(figure 7). Another polymorphism, 184F, was not selected by prior therapy with AQ, and two other mutations, *1034C* and *1042N*, were not seen in any samples. We did not study associations with the other polymorphism linked to AQ sensitivity, *pfcrt 76T*, as this mutation has been universally seen in *P.falciparum* samples assessed in Kampala in recent years, including a random collection of 100 samples from this cohort (unpublished observation).

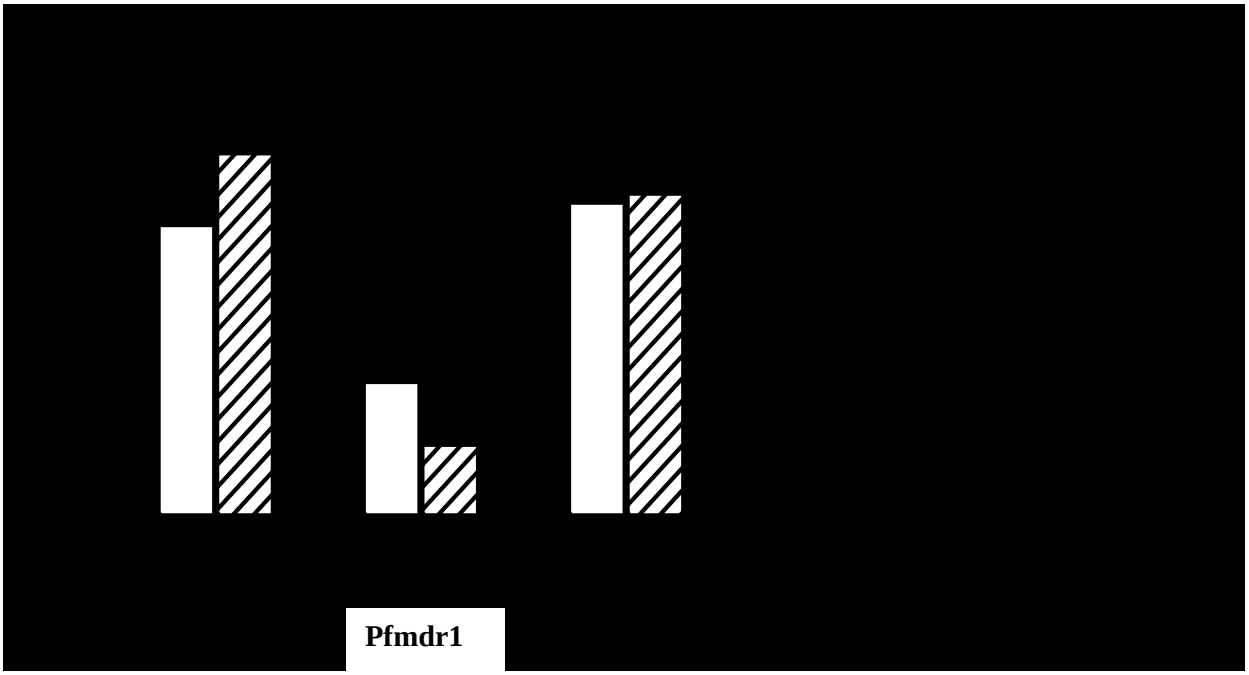


Figure 7 Selection of *pfmdr-1* alleles.
The proportions of samples with mutations at the alleles indicated are shown for the two comparisons of prior and no prior amodiaquine

5.3.4 Association of parasite genetic polymorphisms and *in vitro* AQ sensitivity

We also attempted to identify associations between *pfmdr-1* polymorphisms and *in vitro* sensitivity to AQ. Straightforward correlations were not seen (figure 8). The prevalence of the mutant allele was slightly higher in parasites categorized as resistant for *N86Y* (prevalence *86Y* 38/48 (79.2%) in sensitive vs. 11/13 (84.6%) in resistant), prevalences were the same for *1246Y* (prevalence *1246Y* 37/48 (77.1%) in sensitive vs. 10/13 (76.9%) in resistant), and the prevalence of the mutant allele was lower in resistant parasites for Y184F (prevalence 184F 9/48 (18.8%) in sensitive vs. 1/13 (7.7%) in resistant). No differences in allele prevalence were statistically significant (Fisher Exact Test).

Figure 8 Correlation of *in vitro* sensitivity to monodesethylamodiaquine MDAQ with *pfmdr-1* genotypes.
For each sample studied, the sequence at the three alleles of interest and the *in vitro* sensitivity to MDAQ are shown. No correlation of in vitro sensitivity to MDAQ with *pfmdr-1* genotypes was significant

5.4 *In vitro* sensitivity to various antimalarials drugs

To determine baseline *in vitro* senstivity patterns of *P. falciparum* fresh clinical isolates in Uganda against various antimalarial drugs, samples were collected from a cohort of children in the Mulago III Parish of Kampala enrolled in a longitudinal drug efficacy comparison (Dorsey and others 2007). The clinical trial began in November, 2004; samples for this study were collected between 2006 and 2008. *HRP-2* assay was used to determine *in vitro* sensitivity of fresh clinical isolates to currently antimalaria recommended in ACT's and other commonly used monotherapy drugs in Kampala. A total of 241 were subjected to *in vitro* sensitivities , and successful responses were seen in 181(75%), 196(81%), 206(86%), 212(88%),200(83%), and 199(83%) samples to chloroquine(CQ), quinine(QN), monodesethylamodiaquine(MDAQ), dihydroartemisinin(DHA), lumefrantrine(LUM) and piperaquine(PIP) respectively. Failure to get test results for a particular sample was due to poor in *vitro* growth, culture contamination, or failure to achieve adequite fit in a log dose curve of IC_{50} data. The test to be valid *HRP-2* assay should full fill the following criteria below: $OD_{max}/OD_{background} \geq 2$, where OD_{max} is the OD in the drug free control well and $OD_{background}$ is the OD in the well with the highest drug concentration at 72hours, OD max – Odbackground $\geq$ 0.4 at 72hours (WHO,2007).Table 7 summarizes the *in vitro* responses of clinical isolates to the above listed antimalarials. DHA and LUM were the most potent drugs with geometric mean of 0 .55nM (range, 0.132 - 4.79 nM) and 0.51 nM (range, 0.186 - 29.37 nM) respectively. The geometric mean IC_{50} values of clinical isolates for other drugs were as follows: MDAQ 66.4 nM (range, 6.5 - 311.6nM); CQ(101.1nM, range, 15.64 – 767.02 nM); QN(94.36 nM, range, 15.39 - 760.87 nM); PIP (6.1 nM, range, 5,45 to 6.83 nM). Corresponding mean IC_{50} nM values for W2and D6 clone for different drugs were as; (CQ580.81, 47.80; QN425.72, 106.94; MDAQ 230.25, 30.85; DHA 0.40, 0.79; LUM 0.39, 1.82; PIP 15.55, 6.26) respectively.

Table 7 *In vitro* sensitivity Geometric mean (GM) IC_{50} (nM) to various antimalarial drugs in Kampala

Anti-malarial	n	% resistance	GM	[95% CI]	Range
Chloroquine (CQ)	181	50.8	101.10	86.84 – 117.71	15.64 – 767.02
Quinine (QN)	196	0	94.36	80.37 – 110.81	15.39 – 760.87
Monodesethylamodiaquine (MDQA)	206	47.5	66.40	58.05 – 75.95	6.5 – 311.61
Dihydroartemisinin (DHA)	212	0	0.55	0.50 – 0.61	0.132 – 4.79
Lumefrantrine (LUM)	200	0	0.51	0.46 – 0.56	0.186 – 29.37
Piperaquine (PIP)	199	0	6.10	5.45 – 6.83	1.56 – 46.16

n =Number of samples analysed.

Proposed cut offs for resistance are: 100 nM for chloroquine, 800 nM for quinine (Basco and Le Bras 1993), (Pradines and others 1999) , (Ringwald and others 1999): 80 nM for desethylamodiaquine, 150nM lumefrantrine, and 10nM dihydroartemisinin(Kaddouri and others 2008). Piperaquine no cut off established.

5.4.1 Relationship between *in vitro* sensitivities of various antimalarial drugs.

Table 8 shows the Pearson's correlation for the *in vitro sensitivities* of different antimalarials. The correlation plot of logCQ IC_{50} values against those for QN and MDAQ showed that the activity of these compounds had a positive association (r=0.4.r and r=0.6, $p<0.001$). Quinine IC_{50} values were weakly and significantly correlated to LUM(r=0.2, $p<0.05$). In case of MDAQ IC_{50} values also had same weak but significant correlation with LUM (r=0.2, $p<0.05$). In contrast, the following pairs had a negative correlation which were significant; CQ/DHA (r= - 0.2, $p<0.04$) and MDAQ/DHA(r= - 0.2, $p<0.001$).

Table 8 Correlation between the *in-vitro* IC50 value

Anti-malarial	Pearson's correlation r	p-value
CQ – QN	0.4	<0.001
CQ – QA	0.6	<0.001
CQ – PIP	0.1	0.1469
CQ – DHA	-0.2	0.0358
CQ – LUM	-0.1	0.1432
QN – QA	0.6	<0.001
QN – DHA	-0.1	0.0928
QN – LUM	0.2	0.0537
QN – PIP	-0.1	0.4900
QA – DHA	-0.2	0.0022
QA – LUM	0.2	0.0462
QA- PIP	0.1	0.1361
DHA – LUM	0.02	0.7949
DHA – PIP	0.07	0.361
LUM – PIP	0.1	0.1204

Table 8. A positive correlation of *in vitro* sensitivities between CQ, MDAQ, and QN (r=0.4-0.6; p<0.001), by Pearson's correlation), but not for other comparisons between non-artemisinins. We could not find a strong relationship between the activities of CQ and other quinoline-based drugs, such as lumefantrine, which belong to the same family as QN. The activities of lumefantrine were negatively correlated to that of CQ.

5.4.2 Association of pfmdr-1 mutations with $1C_{50}$s

We went further as shown in table 11 to analyze the prevalences of 5 *pfmdr- l* mutations associated with antimalarial resistance for parasites that were the most and least sensitive to 5 drugs of particular interest. In general there was equal distributions of *84Y, 1246Y* and *184F* mutation in each group but considering common *pfmdr-1* polymorphisms, parasites most sensitive to MDAQ, QN, and LUM, but not PIP, were more likely to have wild type sequence at allele *86N*; there were no clear associations at *184F* or *1246Y,*

Table 9 Distribution of pfmdr-*1* mutations mediating antimalarial resistance with$1C_{50}$s

			***Pfmdr-1* gene mutations associated with resistance**								
			N86Y			**D1246Y**			**Y184F**		
Drugs	N=42	Range of $1C_{50}$ nM	Wild type	mutant	Mixed	Wild type	mutant	Mixed	Wild type	mutant	mixed
CQ	n=20	324.20 – 767.03	0	20	0	2	8	10	17	2	1
	n=20	15.65 – 49.03	5	9	6	7	10	3	18	2	0
QN	n=20	379.81 – 760.87	2	14	4	3	12	5	19	1	0
	n=20	15.4 – 98.80	6	11	3	10	6	4	16	4	0
DMAQ	n=20	199.18 – 311.61	0	17	3	0	15	5	19	1	0
	n=20	6.25 – 18.95	6	12	2	6	8	6	15	5	0
LUM	n=4	12.51 – 29.37	0	4	0	2	2	0	4	0	0
	n=4	0.19 – 0.23	2	1	1	2	1	1	4	0	0
PIP	n=18	19.56 – 38.85	4	12	2	4	10	4	15	3	0
	n=18	1.56 – 1.96	5	11	2	5	9	4	13	4	1

n=Number of samples analysed

5.4.3 Change of *in vitro* sensitivities to various antimalarials between 2006 – 2008

Figure 9 shows scatter plots depicting the changes of sensitivities of various drugs during the period of 2006 - 2008. Drugs like CQ, QN and AQ showed wider variations as compared to DHA, PIP and LUM. We had an important limitation, as no reference parasite strains were assessed over the course of this comparison; reference strains were only obtained later. Nevertheless, it was of value to compare geometric mean IC_{50}'s between 2007 to 2008 (table 9). We found significant decrease of sensitivities with *P.falciparum* strains with CQ, MDAQ, and QN, but not with DHA, LUM and PIP.

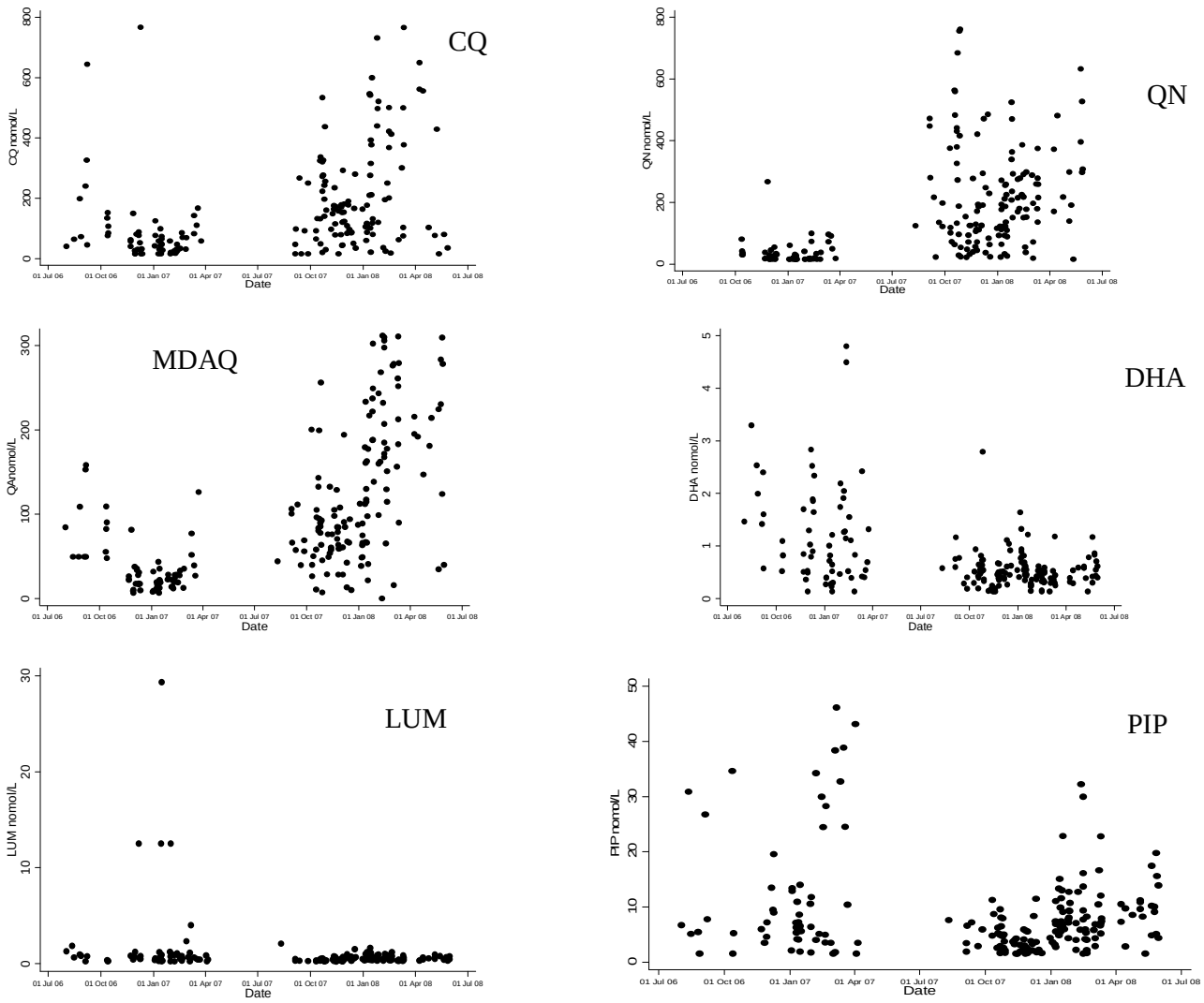


Figure 9 Shows scatter plots depicting the changes of sensitivities of various drugs during the period of 2006- 2008.

Drugs like CQ, QN and AQ showed wider variations as compared to DHA, PIP and LUM. We had an important limitation, as no reference parasite strains were assessed over the course of this comparison; reference strains were only obtained later. Nevertheless, it was of value to compare geometric mean $IC_{50's}$ between 2007 to 2008 (table 10). We found significant decrease of sensitivities with P.falciparum strains with CQ,MDAQ, and QN, but not with DHA, LUM and PIP.

Table 10. Change of *in vitro* sensitivities to various antimalarials between 2006 – 2008

Anti-malarial	2007		2008		p-value
	n	GM IC_{50} nM	n	GM IC_{50} nM	N
Chloroquine	98	120.84	53	269.28	<0.001
Quinine	102	152.7	72	215.14	0.0115
Monodesethylamodiaquine	100	61.15	77	166.56	<0.001
Dihydroartemisinin	100	0.72	83	0.52	0.0165
Lumefantrine	98	1.08	84	0.55	0.162
Piperaquine	99	7.85	82	8.93	0.3714

Geometric mean=GM, n=sample size, IC_{50} =50% of parasited are killed by the drug

Table 10.The geometric mean $IC_{50's}$ between 2007 to 2008 are shown above and we found significant decrease of sensitivities with P.falciparum strains with CQ,MDAQ, and QN, but not with DHA, LUM and PIP.

CHAPTER SIX

DISCUSSION

This study was carried out between 2005 – 2008, assessed changes in polymorphisms of a drug resistant alleles of *pfmdr-1* and *pfcrt* halotypes between baseline and new infections during therapy with AS/AQ treatment arm. ,We developed systems for studying fresh *P. falciparum* clinical isolates and evaluated changes in complexity of infection during short time culture. In addition, assessed the impact of various amodiaquine containing regimens on the *in vitro* sensitivity of recurrent *P. falciparum* isolates. Lastly, evaluated associations between the *in vitro* drug sensitivity and genotypes of isolates and clinical outcomes after antimalarial therapy.We report from this study that although artemisinin derivatives are effective in clearing malaria parasites, ACTs select *86Y pfmdr-1* mutation in re-infections that may be associated with decreased response to AQ. Secondly, initial genotypes offer an imperfect representation of clinical complexity of infection, and loss of strains in culture may be due to diminished fitness of some drug resistant strains. Our results further reveals the concern regarding the long-term prospects for AQ-containing combination therapies for the treatment of *P. falciparum* malaria, by selection of resistant parasites that was readily apparent after prior therapy with AQ. We were able to develop malaria culture laboratory for the first time in Uganda which is being used for *in vitro* sensitivities studies and molecular studies. Lastly, we generated baseline *in vitro* sensitivity results for recommended antimalarial use Uganda which may offer the best means of tracking emerging resistance to ACTs, as there are no confirmed molecular markers of resistance to ACTs, and clinical trials may not be sensitive enough to detect early resistance to combination regimens.

Selection of *Plasmodium falciparum* resistance-mediating *pfcrt* and *pfmdr-1* alleles after treatment with artesunate-amodiaquine in Tororo Uganda

In order to assess changes in occurrence of various polymorphisms of a drug resistant alleles of *pfmdr-1 and pfcrt* between baseline and new infections during AS/AQ therapy, we utilized samples from a clinical trial comparing the efficacies of AL and AQ/AS in Tororo, Uganda (Bukirwa and others 2006). Children aged 1 to 10 years with uncomplicated malaria were randomized to receive directly observed therapy with one of the ACTs and followed for 28 days. Our clinical trial identified no recrudescences, but frequent new infections (66% of subjects) within a month after treatment of uncomplicated malaria. We evaluated polymorphisms at the *pfmdr-1* alleles (*N86Y, Y184F, S1034C, N1042D, D1246Y*) in 201 isolates collected before the initiation of treatment representing all patients randomized to therapy with (AS/AQ) and 132 isolates from all new infections that presented over 28 days of follow-up after therapy. We also evaluated *pfcrt* haplotypes at positions 72-76 in 90 randomly selected pretreatment and 90 randomly selected new infection isolates. Isolates from the study for selection of *Plasmdium falciparum* reistance- mediating alleles after treatment with artesunate-amodiaquine in Uganda, had high prevalence of *pfmdr-1 86Y* and *1246Y* mutations, and prevalence was greater in new isolates after therapy with AS/AQ than in isolates collected before therapy. This finding is, consistent with studies of AQ monotherapy (Holmgren and others 2006) from other regions, the AQ containing regimen selected for the *86Y* mutation(Zongo and others 2007b). In Burkina Faso, the presence of the *86Y* mutation prior to treatment predicted failure of AQ monotherapy, suggesting that selection of mutant parasites will lead to an increased likelihood of drug resistance. Another study, from Sudan, did not identify associations between the *86Y* allele and AQ treatment outcomes, but this study assessed only 14-day treatment outcomes and did not evaluate selection of mutant parasites after therapy Thus studies in Tanzania(Sisowath and others 2005), Uganda (Dokomajilar and others 2006a), and (Zongo and others 2007a),Burkina Faso showed

selection of *pfmdr-1* wild type N86 and/or D1246 alleles after treatment with AL, presumably due to selective pressure of lumefantrine. This study showed the opposite selection with AL. As the *pfcrt* 76T mutation that mediates CQ resistance was universal in our isolates, we could not identify associations between *pfcrt* alleles and AQ treatment outcomes, as seen in sites with lower prevalence of the mutant parasites, where *pfcrt* 76T predicted AQ treatment failure (Ochong and others 2003)**,** (Dokomajilar and others 2006a).

We went further to characterize *pfcrt* haplotypes because they affect the sensitivity to CQ and AQ. The two haplotypes, *CVIET* from Asian and African isolates and *SVMNT* from South America have been associated with CQ resistance(Cortese and others 2002; Vieira and others 2004; Voruganti and others 2002). Additional studies (Dittrich and others 2005; Warhurst 2003) reported that the *SVMNT* haplotype found in Southeast Asia appeared to be associated with a decreased efficacy of amodiaquine (AQ). Though, (Sidhu and others 2002) reported that parasites transfected with *pfcrt* with the SVMNT haplotype have decreased sensitivity to AQ and its active metabolite, so if parasites of this haplotype are circulating, as was recently demonstrated in Tanzania by(Alifrangis and others 2006), they might be selected by AS/AQ. In our study all isolates contained the CVIET haplotype. This implies that selection of those *pfcrt* haplotypes is not yet on going in Tororo Uganda.

Complexity of *Plasmodium faciparum* clinical samples during short-term culture in Kampala Uganda

Culture of fresh parasites is widely used to study the *in vitro* drug sensitivity of infecting parasites and to search for associations between parasite features and clinical outcomes. However *P. falciparum* infections are commonly polyclonal in many areas, and, since not all parasites successfully grow in culture, it is unclear how well *in vitro* culture represents the complexity of clinical infections. To characterize changes in COI during short-term culture of *P. falciparum*, samples were collected from a cohort of children in the Mulago III Parish of Kampala enrolled in a longitudinal drug efficacy comparison (Dorsey and others 2007). The clinical trial began in November, 2004; samples for this study were collected between October, 2005 and May, 2006. At the time of new diagnosis of malaria blood was spotted onto filter. Giemsa-stained thin blood smears were examined, and if *P. falciparum* infection then culture was initiated. Giemsa-stained smears of cultures were examined daily and at the same time, blood was spotted onto filter paper for molecular studies for period of nine days. A total of 211 samples were cultured from children in Kampala (aged 1 – 10 years at the time of study enrollment) with acute malaria. Complexity of infection was measured by assessment of the diversity of highly polymorphic *P. falciparum* genes, most commonly that encoding merozoite surface protein-2 (*msp-2*). Of the samples collected, 98(46%) were successfully cultured for at least 9 days. The measured complexity of infection was lower than that determined in a number of other areas of Africa (Bray and others 1996a)**,** (Alifrangis and others 2006), and also lower than the COI of 2.88 that we measured in children in Kampala in a prior study (Duraisingh and others 2000a). The relatively low COI reflects the fact that Kampala has relatively low transmission for sub-Saharan Africa. In addition, our genotyping was based on only a single polymorphic gene, likely underestimating the true diversity of cultured parasites. Lastly, study children had benefited from prompt treatment of all malaria episodes for 6 months or more prior to evaluation for

this study, likely decreasing parasite prevalence. To gain insight into the sensitivity of single time point assessment of COI, we followed the appearance of new strains after Day 0. Strains not present on Day 0 were first identified on Days 1-5; 26 new strains in 20 cultures were identified (figure 3B). Based on these results, the Day 0 genotype understated the true COI in 20% of samples. The appearance of new strains identifies limitations in the sensitivity of a single *msp-2* assessment in antimalarial drug efficacy studies. Prior studies have shown changes in parasite genotypes within hours after treatment in France (Jafari and others 2004), quite constant genotypes in Sweden (Farnert and Bjorkman 2005), and a moderate level of change in genotype between Day 0 and Day 1 in Tanzania (Martensson A et al 2007). Since all parasites that appear in culture after Day 0 must necessarily have been present initially, our results identify the minimum number of circulating strains that were missed by single time point Day 0 genotyping. Our results offer, to our knowledge, the first assessment of COI during culture of freshly isolated malaria parasites. They have a number of ramifications. First, we confirm studies from other areas showing that COI is underestimated by a single characterization on Day 0, presumably due to limits in sensitivity for low-abundance strains. This observation is particularly relevant for drug efficacy trials. In this case, strains missed on Day 0 and seen after treatment will be misclassified as new infections, thus understating levels of drug resistance. In our study, 26 strains appeared only after Day 0; mostly on day 1, suggesting that genotyping using a combination of Day 0 and Day 1 patterns as baseline, as suggested previously (Martensson and others 2007) , might increase the reliability of assessments of recrudescence rates, albeit at the cost of added complexity in trial design. Second, we characterize the dynamics of parasite complexity in fresh cultures. Many samples did not survive in culture for 9 days, and surviving cultures showed a gradual decrease in COI over time. Thus, parasites studied after culture adaptation represent only a subset of those infecting a patient, and inferences regarding associations between cultured parasites and

clinical outcomes must consider the possibility that some disease-mediating strains may be lost before analysis. Third, we offer the intriguing possibility that loss of some strains in culture is due to a relative disadvantage of drug resistant parasites. Thus, evaluation of parasites after culture adaptation may include a bias toward drug sensitive parasites. We examined the possibility that parasite fitness, considered here as the ability of a parasite to survive in culture, played a role in the disappearance of certain strains. Fitness might be diminished in drug-resistant parasites, as appears to be the case with chloroquine-resistant strains, which were replaced by wild type parasites after drug pressure was removed in China and Malawi (Laufer and others 2006) . We therefore evaluated three resistance-mediating polymorphisms in mixed cultures that subsequently became monoclonal (within our limits of detection): the key mediators of antifolate resistance in East Africa, *dhfr C59R* and dhps *K540E* (Dorsey and others 2004) , and a mediator of responses to a number of antimalarials, *pfmdr-1 N86Y*. Thus, for the mutations that mediate SP resistance, when cultures became monoclonal, wild type parasites persisted in 6 of 7 instances. Of note, the *pfmdr-1 86Y* genotype, which is typically classified as mutant, actually leads to improved activity of a number of drugs, including mefloquine, quinine, and artemisinins (Pickard and others 2003). Thus it is plausible that all three studied polymorphisms may have come at some cost to fitness, as suggested by our results showing parasites with *dhfr C59, dhps K540,* and *pfmdr-1 86Y* out competing those with other sequences at these loci in culture. Of note, an important factor not considered in our *in vitro* studies is the impact of circulating drugs on the removal of drug-sensitive parasites. Our study had some limitations. First, it was limited to parasites that were successfully cultured for 9 days. For about half of our cultures, no strain survived for 9 days. Factors leading to rapid loss of these strains were not considered. Changes in techniques, including use of a defined gas mixture, increase in concentration of the serum substitute, or use of human serum might have improved culture survival. Second, we used

only a single marker, *msp-2*, to distinguish parasite strains. The *msp-2* marker has previously been shown to provide reasonable discrimination of *P. falciparum* strains in Kampala (Cattamanchi and others 2003), although consideration of additional strains would likely increase estimates of COI. Third, we considered genotypes based on amplification of parasite DNA from dried blood spots. This technique matched that used in most clinical trials, but might not be as sensitive as amplification of DNA purified from whole blood, again potentially underestimating COI. Fourth, for associations between parasite polymorphisms and survival in culture, our samples sizes were small, as relatively few cultures underwent changes in population structure, and our knowledge of the impact of polymorphisms on parasite fitness is incomplete. Despite these limitations, our results offer insight into the dynamics of freshly prepared *P. falciparum* cultures.

Selection of parasites with diminished drug sensitivity by amodiaquine containing antimalarial regimens in Uganda

We further analyzed selection of parasites with diminished *in vitro* sensitivity by amodiaquine – containing antimalarial regimens in Uganda. ACT is now the international standard for the treatment of *P. falciparum* malaria, as multiple new combination regimens offer excellent antimalarial efficacy. However, there is concern that, since all ACTs include a short-acting artemisinin and long-acting partner drug, the regimens will select for resistance to partner drugs, especially in areas where reinfection after treatment is common. This concern is arguably greatest for AQ-containing combinations, as resistance to this drug is already fairly common. AQ is rapidly metabolized to MDAQ , an active metabolite which has a half life of about 2 weeks . Thus, after therapy with a regimen containing AQ, MDAQ circulates at decreasing levels for many weeks. We hypothesized that children treated with AQ-containing regimens would be at increased risk of AQ-resistance in subsequent infections. To test this hypothesis, we studied *in vitro* sensitivity to MDAQ in *P. falciparum*

parasites causing uncomplicated malaria in a cohort of children in Kampala who received AQ/SP, AQ/AS, or AL for each episode of uncomplicated malaria(Dorsey and others 2007). Kampala is known to have a fairly high prevalence of AQ-resistant malaria(Staedke and others 2001) and parasites that caused malaria in our cohort demonstrated a wide range of sensitivities to MDAQ. Importantly, parasites that caused infections within 12 weeks of a prior treatment with AQ had decreased sensitivity to MDAQ compared to those that caused infections in individuals not recently treated with AQ. Thus, as we hypothesized, prior treatment with AQ selected for parasites with diminished drug sensitivity. This result suggests that resistance to AQ may quite rapidly be selected by treatment of malaria with combinations including AQ. We identified a broad range of *in vitro* sensitivities to MDAQ in parasites causing uncomplicated malaria in our cohort of Ugandan children. Parasites with high IC_{50}'s that might mediate clinical resistance were seen in 21% of infections, consistent with prior clinical trials in Kampala showing frequent treatment failures with AQ monotherapy . These results suggest a more extensive problem with AQ resistance in Uganda than in some other regions of Africa, where >90% of parasites were sensitive to MDAQ *in vitro* in recent studies in Madagascar(Rason and others 2007), Ghana(Quashie and others 2007), Cameroon(Basco and Ringwald 2007), Congo(Pradines and others 2006), Senegal(Agnamey and others 2006), Rwanda(Tinto and others 2006) and in a collection of isolates from different countries. Older studies that considered sensitivity to AQ, rather than MDAQ, also generally reported high levels of sensitivity, although 16% of samples were reported to demonstrate in vitro resistance in a study from the Central African Republic (Menard and others 2005). In our study, MDAQ-resistant parasites caused infections in those with and without recent prior therapy with AQ; selective pressure from circulating AQ was not required for infection with a resistant strain. However, prior therapy with AQ led to an increased predilection for infection with MDAQ-resistant parasites.

It was also of interest to determine if the *in vitro* sensitivity of parasites to MDAQ was associated with clinical outcomes. However, of the 61 parasite samples which were successfully studied, only 7 had recrudescent infections as determined by multi-locus genotyping, and only 4 of these were in subjects in an AQ-containing treatment arm. Therefore we lacked power to assess associations between *in vitro* sensitivity to MDAQ and treatment outcomes. Nonetheless, recrudescences occurred after infection with parasites sensitive and resistant to MDAQ, arguing against any simple association between *in vitro* drug sensitivity and clinical response. Similarly, in a study in Gabon, *in vitro* sensitivity to MDAQ did not correlate with clinical response to therapy with AQ(Aubouy and others 2004). Indeed, it is likely that clinical responses to AQ-containing combination regimens are complex, with effects of varied pharmacokinetics(Krishna and White 1996), pharmacogenomics(Parikh and others 2007), host immunity, and other factors in addition to the drug responsiveness of parasites. Nonetheless, parasite drug sensitivity is clearly an important component of a successful treatment response. A better appreciation of mediators of parasite resistance to MDAQ will be of value in optimizing utilization of available drug regimens.

We also searched for associations between *in vitro* drug sensitivity and polymorphisms that have previously been linked to altered sensitivity to MDAQ. The *76T* polymorphism in the putative drug transporter *pfcrt*, which is the primary mediator of resistance to chloroquine, also predicts poor response to AQ (Ochong and others 2003) (Dokomajilar and others 2006a) (Happi and others 2006) and is selected in new infections following therapy with AQ(Zongo and others 2007b) (Dokomajilar and others 2006a) (Happi and others 2006) (Holmgren and others 2006) (Djimde and others 2008). The *pfcrt 76T* mutation was also associated with decreased in vitro sensitivity to MDAQ in field isolates from the Central African Republic(Menard and others 2006) and in genetically altered laboratory strains(Sidhu and

others 2002). Considering our results, it is noteworthy that, even with likely 100% prevalence of *76T* in our set of parasites, 79% of samples demonstrated sensitive *in vitro* responses to MDAQ. Thus, the common *pfcrt* polymorphism, although predictive of decreased response to MDAQ, does not by itself dictate MDAQ resistance. Polymorphisms in a second putative drug transporter, *pfmdr-1*, have not been as clearly linked to AQ treatment outcome, but in some studies from Africa the *pfmdr-1 86Y* polymorphism predicted poor AQ treatment outcomes(Dokomajilar and others 2006a) (Happi and others 2006). Also, as seen in this study, *pfmdr-1* 86Y was selected by prior therapy with AQ in a number of studies(Zongo and others 2007a) (Djimde and others 2008; Dokomajilar and others 2006a) (Happi and others 2006) (Holmgren and others 2006) (Nsobya and others 2007). However, prevalence of the *pfmdr-1* 86Y mutation was not associated with *in vitro* drug sensitivity in samples from the Central African Republic(Menard and others 2006), from Colombia(Echeverry and others 2007), or in our study from Uganda. Taken together, our results are consistent with those from other areas, suggesting contributions of polymorphisms in both *pfcrt* and *pfmdr-1* to AQ resistance, but likely involvement of additional host (e.g. genetics, pharmacokinetics, immunity) and parasite (e.g. additional polymorphisms) factors in high level AQ resistance.

The study had some limitations. The evaluations of *in vitro* sensitivity of clinical samples were necessarily limited to parasites capable of growing to allow measurement of *in vitro* drug sensitivity. Infections in Kampala are commonly polyclonal. Most of our *in vitro* sensitivity measurements were thus based on assessment of a mixed population of parasites in which competition between sensitive and resistant strains might obscure results for parasites capable of mediating resistant outcomes. Comparisons with results
from other studies must take into account the fact that a number of different assays have been used to measure *in vitro* drug sensitivity; comparisons of values between studies may be misleading. *In vitro* measurements leave potential for error, as, since they are measured only

during the first life cycle after parasite collection, they cannot be repeated. Thus, for any single measurement, there is the possibility that human error or other factors led to misrepresentation of the true results. Despite these limitations, considering our large set of evaluated samples, our results strongly suggest that prior therapy with AQ selects for decreased sensitivity to the drug.

Evaluation of *in vitro* drug sensitivity of chloroquine, quinine, desethylamodiaquine, dihydroartemisinin, lumefantrine and piperaquine in Kampala Uganda

Assessment of the *in vitro* sensitivity of malaria parasites to drugs offer the best means of tracking emerging resistance to ACTs, as there are no confirmed molecular markers of resistance to ACTs, and clinical trials may not be sensitive enough to detect early resistance to combination regimens. We studied the *in vitro* senstivity of *P. falciparum* to antimalarial drugs to provide information on resistance patterns in samples from a cohort of children in the Mulago III Parish of Kampala enrolled in a longitudinal drug efficacy comparison (Dorsey and others 2007) . The clinical trial began in November, 2004; samples for this study were collected between 2006 and 2008. *HRP-2* assay was used to determine *in vitro* sensitivity. A total of 241 were subjected to *in vitro* sensitivities,and successful responses were seen in 181(75%), 196(81%), 206(86%), 212(88%),200(83%),and199(83%)sample to chloroquine(CQ),quinine(QN),monodesethylamodiaquine(MDAQ),dihydroartemisinin(DHA), lumefantrine(LUM) and piperaquine(PIP) respectively. Recent clinical trials have shown poor efficacy of chloroquine in different areas of Uganda, This clinical finding is not supported by our *in vitro results* which show a prevalence of 50.8% chloroquine resistance using a cut – off of 100nM, and the presence of 100% of *76T*. The cause of low prevalence of in *in vitro* results may be due to the presenceof mixed population of parasites sensitive and resistant strains in which the latter IC_{50} may obscure results for parasites capable of mediating resistant or new compensatory mutations *in vitro* such as *S163R,* which may counteract the

K76T substitution as reported by(Durand and others 2001; Johnson and others 2004; Sarr and others 2005; Thomas and others 2002; Vinayak and others 2003).

The study also reports geometric mean IC_{50}'s for monodethylamodiaquine of 66.4 nmol/liter; range, 6.5 to 311.61 nmol/liter) with phenotypic profiles of some isolates tested against above the estimated cut-offs resistance (80nM)(Kaddouri and others 2008). This results show prevalence of 47.5% above the the cut – off values. Our results are consistent with clinical efficacy studies which have reported AQ monotherapy resistance in Kampala Uganda than in some other regions of Africa, where >90% of parasites were sensitive to MDAQ *in vitro* in recent studies in Madagascar, Ghana, Cameroon , Congo, Senegal, Rwanda. This result suggests that resistance to AQ may quite rapidly be selected by treatment of malaria with combinations including AQ thus a need to monitor it's sensitivity regularly in Uganda.

Comparing with the other antimalarial drugs tested, we found that *P. falciparum* clinical isolates were highly sensitive to dihydroartemisinin, with geometric mean IC_{50} of 0 .55nM; (range, 0.132 to 4.79 nmol/liter). Our results are consistent with other studies from Africa parasites with 0.58,0.73,2.2,0.85, 1.1,2.6 nM from Sao Tome, Gabon, Senegal, Bangui and Rwanda respectively(Ramharter and others 2002), (Henry and others 2006), (Ferreira and others 2007) Isabel et al.,2008 ; (Menard and others 2005); (Tinto and others 2006). In contrast, a trend of towards significantly low tolerance of geometric mean $IC_{50,}$s of artemisinin derivatives of 9nM and 40nM were recently reported from Laos, and French Guiana respectively(Mayxay and others 2007) and (Jambou and others 2005).

A study on field isolates from Madagascar reported IC_{50}s of piperaquine which was widely dispersed, ranging from <12.5 nmol/liter to >250 nmol/liter, with values of <100 nmol/liter for the majority (83%) of isolates (Le Bras and others 1983). By contrast, in our study piperaquine was highly active (geometric mean 50% inhibitory concentration, of6.1

nmol/liter; range, 5.45 to 6.83 nmol/liter) within a very narrower range of *in vitro* sensitivities values (1.56 to 46.16 nmol/liter).

In Mali and Lao reported slightly higher $1C_{50}$s of lumefantrine (11.2 nmol/liter; range, 2.9 to 49.3 nmol/liter) and ((59.07 nmol/liter; range, 4.4 to 251 nmol/liter) by (Kaddouri and others 2008) and (Mayxay and others 2007)respectively. Our study showed lumefantrine was highly active (geometric mean 50% inhibitory concentration, 0.51 nmol/liter; range, 0.186 to 29.37 nmol/liter). These results are comparable to findings in Cameroon, (8.27 nmol/liter; range, 1.67 to 22.8 nmol/liter) were chloroquine susceptible isolates and chloroquine resistance strains(10.2 nmol/liter; range, 4.41 to 25.2 nmol/liter)(Basco and Ringwald 2007) were all sensitive to the drug. Thus need to monitor the level of lumefantrine regularly in Uganda.

In the case of quinine, isolates had (geometric mean 50% inhibitory concentration, 94.36 nmol/liter; range, 15.64 to 760.87 nmol/liter) and some few isolates were close to threshold cut off 800nM(Ringwald and others 1999). Qur results are similar to those reported in Rwanda(Tinto and others 2006)(153.5 nmol/liter; range, 13.4 to 793.6 nmol/liter), where all 74 isolated were susceptible to quinine. In case of using lower threshold of 500nM recommended by (Pettinelli and others 2004) 0.04% of our results would have been above that value .

There are multiple limitations with *in vitro sensitivity* studies; differences in results between these *in vitro* studies may be due to methodological differences rather than inherent differences between isolates. In addition, physiological differences between isolates that alter their basal level of susceptibility; single versus mixed infections; differences in rates of growth of non adapted isolates under *in vitro* conditions; experimental variability within wells; and the degree genetic complexity of the resistance phenotypes may affect in vitro IC50 measurements. Furthermore, *in vitro* assays of a single fixed drug combination are

usually difficult to interpret since sensitivities are measured aqainst individual drugs. Having said that, our results for control reference strains were consistent with prior findings for these strains, suggesting validity for our *in vitro* sensitivity assessments. Despite these technical limitations, *in vitro* drug susceptibility assay remains an important component of research and surveillance of malaria.

Cross – resistance patterns between atimalarial drugs

We also report a positive correlation of *in vitro* sensitivities between CQ, MDAQ, and QN (r=0.4-0.6; p<0.001), by Pearson's correlation), but not for other comparisons between non-artemisinins. Our results are consistent with results published by(Bray and others 1996b) and (Draper and others 1988). However, in the present study, we could not find a strong relationship between the activities of CQ and other quinoline-based drugs, such as lumefantrine, which belong to the same family as QN. The activities of lumefantrine were negatively correlated to that of CQ as reported by Standwell et al., 2007).

A positive correlation was seen between AQ and QN, which is consistent with what was published by(Pradines and others 1998) (Afonso and others 2006). There was a significant but low correlation of response between chloroquine and piperaquine(r = 0.1, P < 0.05) in our study as compared to (r = 0.257, P < 0.05)(Basco 2003). Thus a need to reemphasize that, given the Chinese experience, piperaquine monotherapy is probably not a viable option for long-term use of this drug and that it should be administered as a combination therapy to maximize its clinical utility.

CHAPTER SEVEN

CONCLUS

ION AND RECOMMENDATION

1. We report that although AS/AQ combination therapy is effective in clearing malaria parasites, in re-infections most of strains selected are associated with decreased response to AQ in Uganda.

2. For the first time in Uganda, we have developed a molecular research laboratory to study fresh clinical isolates of *P. falciparum* by performing culture, *in vitro* culture and molecular studies.

3. To the best our knowledge, this is the first assessment of complexity of infection during *in vitro* culture of freshly isolated malaria parasites. In drug efficacy trails, strains missed on day 0 and seen after treatment will be misclassified as new infections, thus understating levels of drug resistance.

4. Loss of strains during culture of freshly isolated malaria parasites may be due to diminished fitness of some drug resistant strains

5. These results suggest diminishing efficacy of AQ-containing combination regimens as they are increasingly used in Uganda.

6. The generated baseline *in vitro sensitivity* data for the first time in Uganda will be used as yard stick to monitor emerging early resistance to ACTs.

Recommendation

There is a need to continue analyzing in vitro sensitivity of malaria parasites to drugs because it offers the best means of tracking emerging resistance to ACTs, as there are no confirmed molecular markers of resistance to ACTs, and clinical trials may not be sensitive enough to detect early resistance to combination regimens.

Future studies will also provide a platform from which to direct future research on parasite transporter proteins and drug resistance. In particular, elucidation of the mechanisms by which genes mediate resistance to multiple drug classes can help to guide efforts to overcome the spread of drug resistance.

CHAPTER 8

REFERENCES

Adagut IS, Warhurst DC. 2001. Plasmodium falciparum: linkage disequilibrium between loci in chromosomes 7 and 5 and chloroquine selective pressure in Northern Nigeria. Parasitology 123(Pt 3):219-24.

Afonso A, Hunt P, Cheesman S, Alves AC, Cunha CV, do Rosario V, Cravo P. 2006. Malaria parasites can develop stable resistance to artemisinin but lack mutations in candidate genes atp6 (encoding the sarcoplasmic and endoplasmic reticulum Ca2+ ATPase), tctp, mdr1, and cg10. Antimicrob Agents Chemother 50(2):480-9.

Agnamey P, Brasseur P, de Pecoulas PE, Vaillant M, Olliaro P. 2006. Plasmodium falciparum in vitro susceptibility to antimalarial drugs in Casamance (southwestern Senegal) during the first 5 years of routine use of artesunate-amodiaquine. Antimicrob Agents Chemother 50(4):1531-4.

Alifrangis M, Dalgaard MB, Lusingu JP, Vestergaard LS, Staalsoe T, Jensen AT, Enevold A, Ronn AM, Khalil IF, Warhurst DC and others. 2006. Occurrence of the Southeast Asian/South American SVMNT haplotype of the chloroquine-resistance transporter gene in Plasmodium falciparum in Tanzania. J Infect Dis 193(12):1738-41.

Anderson TJ, Nair S, Qin H, Singlam S, Brockman A, Paiphun L, Nosten F. 2005. Are transporter genes other than the chloroquine resistance locus (pfcrt) and multidrug resistance gene (pfmdr) associated with antimalarial drug resistance? Antimicrob Agents Chemother 49(6):2180-8.

Anderson TJ, Roper C. 2005. The origins and spread of antimalarial drug resistance: lessons for policy makers. Acta Trop 94(3):269-80.

Ashley EA, White NJ. 2005. Artemisinin-based combinations. Curr Opin Infect Dis 18(6):531-6.

Aubouy A, Mayombo J, Keundjian A, Bakary M, Le Bras J, Deloron P. 2004. Short report: lack of prediction of amodiaquine efficacy in treating Plasmodium falciparum malaria by in vitro tests. Am J Trop Med Hyg 71(3):294-6.

Babiker HA, Pringle SJ, Abdel-Muhsin A, Mackinnon M, Hunt P, Walliker D. 2001. High-level chloroquine resistance in Sudanese isolates of Plasmodium falciparum is associated with mutations in the chloroquine resistance transporter gene pfcrt and the multidrug resistance Gene pfmdr1. J Infect Dis 183(10):1535-8.

Bacon DJ, Latour C, Lucas C, Colina O, Ringwald P, Picot S. 2007. Comparison of a SYBR green I-based assay with a histidine-rich protein II enzyme-linked immunosorbent assay for in vitro antimalarial drug efficacy testing and application to clinical isolates. Antimicrob Agents Chemother 51(4):1172-8.

Baker J, McCarthy J, Gatton M, Kyle DE, Belizario V, Luchavez J, Bell D, Cheng Q. 2005. Genetic diversity of Plasmodium falciparum histidine-rich protein 2 (PfHRP2) and its effect on the performance of PfHRP2-based rapid diagnostic tests. J Infect Dis 192(5):870-7.

Basco LK. 2003. Molecular epidemiology of malaria in Cameroon. XVI. Longitudinal surveillance of in vitro pyrimethamine resistance in Plasmodium falciparum. Am J Trop Med Hyg 69(2):174-8.

Basco LK, Le Bras J. 1993. In vitro activity of artemisinin derivatives against African isolates and clones of Plasmodium falciparum. Am J Trop Med Hyg 49(3):301-7.

Basco LK, Marquet F, Makler MM, Le Bras J. 1995. Plasmodium falciparum and Plasmodium vivax: lactate dehydrogenase activity and its application for in vitro drug susceptibility assay. Exp Parasitol 80(2):260-71.

Basco LK, Ringwald P. 2007. Molecular epidemiology of malaria in Cameroon. XXIV. Trends of in vitro antimalarial drug responses in Yaounde, Cameroon. Am J Trop Med Hyg 76(1):20-6.

Bray PG, Hawley SR, Mungthin M, Ward SA. 1996a. Physicochemical properties correlated with drug resistance and the reversal of drug resistance in Plasmodium falciparum. Mol Pharmacol 50(6):1559-66.

Bray PG, Hawley SR, Ward SA. 1996b. 4-Aminoquinoline resistance of Plasmodium falciparum: insights from the study of amodiaquine uptake. Mol Pharmacol 50(6):1551-8.

Bray PG, Martin RE, Tilley L, Ward SA, Kirk K, Fidock DA. 2005. Defining the role of PfCRT in Plasmodium falciparum chloroquine resistance. Mol Microbiol 56(2):323-33.

Bukirwa H, Yeka A, Kamya MR, Talisuna A, Banek K, Bakyaita N, Rwakimari JB, Rosenthal PJ, Wabwire-Mangen F, Dorsey G and others. 2006. Artemisinin combination therapies for treatment of uncomplicated malaria in Uganda. PLoS Clin Trials 1(1):e7.

Cattamanchi A, Kyabayinze D, Hubbard A, Rosenthal PJ, Dorsey G. 2003. Distinguishing recrudescence from reinfection in a longitudinal antimalarial drug efficacy study: comparison of results based on genotyping of msp-1, msp-2, and glurp. Am J Trop Med Hyg 68(2):133-9.

Cojean S, Noel A, Garnier D, Hubert V, Le Bras J, Durand R. 2006. Lack of association between putative transporter gene polymorphisms in Plasmodium falciparum and chloroquine resistance in imported malaria isolates from Africa. Malar J 5:24.

Cooper RA, Ferdig MT, Su XZ, Ursos LM, Mu J, Nomura T, Fujioka H, Fidock DA, Roepe PD, Wellems TE. 2002. Alternative mutations at position 76 of the vacuolar transmembrane protein PfCRT are associated with chloroquine resistance and unique stereospecific quinine and quinidine responses in Plasmodium falciparum. Mol Pharmacol 61(1):35-42.

Cooper RA, Hartwig CL, Ferdig MT. 2005. pfcrt is more than the Plasmodium falciparum chloroquine resistance gene: a functional and evolutionary perspective. Acta Trop 94(3):170-80.

Corbett Y, Herrera L, Gonzalez J, Cubilla L, Capson TL, Coley PD, Kursar TA, Romero LI, Ortega-Barria E. 2004. A novel DNA-based microfluorimetric method to evaluate antimalarial drug activity. Am J Trop Med Hyg 70(2):119-24.

Cortese JF, Caraballo A, Contreras CE, Plowe CV. 2002. Origin and dissemination of Plasmodium falciparum drug-resistance mutations in South America. J Infect Dis 186(7):999-1006.

Davis TM, Hung TY, Sim IK, Karunajeewa HA, Ilett KF. 2005. Piperaquine: a resurgent antimalarial drug. Drugs 65(1):75-87.

Desakorn V, Silamut K, Angus B, Sahassananda D, Chotivanich K, Suntharasamai P, Simpson J, White NJ. 1997. Semi-quantitative measurement of Plasmodium falciparum antigen PfHRP2 in blood and plasma. Trans R Soc Trop Med Hyg 91(4):479-83.

Desjardins RE, Canfield CJ, Haynes JD, Chulay JD. 1979. Quantitative assessment of antimalarial activity in vitro by a semiautomated microdilution technique. Antimicrob Agents Chemother 16(6):710-8.

Dittrich S, Alifrangis M, Stohrer JM, Thongpaseuth V, Vanisaveth V, Phetsouvanh R, Phompida S, Khalil IF, Jelinek T. 2005. Falciparum malaria in the north of Laos: the occurrence and implications of the Plasmodium falciparum chloroquine resistance transporter (pfcrt) gene haplotype SVMNT. Trop Med Int Health 10(12):1267-70.

Djimde A, Doumbo OK, Cortese JF, Kayentao K, Doumbo S, Diourte Y, Dicko A, Su XZ, Nomura T, Fidock DA and others. 2001. A molecular marker for chloroquine-resistant falciparum malaria. N Engl J Med 344(4):257-63.

Djimde AA, Fofana B, Sagara I, Sidibe B, Toure S, Dembele D, Dama S, Ouologuem D, Dicko A, Doumbo OK. 2008. Efficacy, safety, and selection of molecular markers of drug resistance by two ACTs in Mali. Am J Trop Med Hyg 78(3):455-61.

Dokomajilar C, Lankoande ZM, Dorsey G, Zongo I, Ouedraogo JB, Rosenthal PJ. 2006a. Roles of specific Plasmodium falciparum mutations in resistance to amodiaquine and sulfadoxine-pyrimethamine in Burkina Faso. Am J Trop Med Hyg 75(1):162-5.

Dokomajilar C, Nsobya SL, Greenhouse B, Rosenthal PJ, Dorsey G. 2006b. Selection of Plasmodium falciparum pfmdr1 alleles following therapy with artemether-lumefantrine in an area of Uganda where malaria is highly endemic. Antimicrob Agents Chemother 50(5):1893-5.

Dorsey G, Dokomajilar C, Kiggundu M, Staedke SG, Kamya MR, Rosenthal PJ. 2004. Principal role of dihydropteroate synthase mutations in mediating resistance to sulfadoxine-pyrimethamine in single-drug and combination therapy of uncomplicated malaria in Uganda. Am J Trop Med Hyg 71(6):758-63.

Dorsey G, Kamya MR, Singh A, Rosenthal PJ. 2001. Polymorphisms in the Plasmodium falciparum pfcrt and pfmdr-1 genes and clinical response to chloroquine in Kampala, Uganda. J Infect Dis 183(9):1417-20.

Dorsey G, Njama D, Kamya MR, Cattamanchi A, Kyabayinze D, Staedke SG, Gasasira A, Rosenthal PJ. 2002. Sulfadoxine/pyrimethamine alone or with amodiaquine or artesunate for treatment of uncomplicated malaria: a longitudinal randomised trial. Lancet 360(9350):2031-8.

Dorsey G, Staedke S, Clark TD, Njama-Meya D, Nzarubara B, Maiteki-Sebuguzi C, Dokomajilar C, Kamya MR, Rosenthal PJ. 2007. Combination therapy for uncomplicated falciparum malaria in Ugandan children: a randomized trial. Jama 297(20):2210-9.

Draper CC, Hills M, Kilimali VA, Brubaker G. 1988. Serial studies on the evolution of drug resistance in malaria in an area of east Africa: findings from 1979 up to 1986. J Trop Med Hyg 91(5):265-73.

Druilhe P, Moreno A, Blanc C, Brasseur PH, Jacquier P. 2001. A colorimetric in vitro drug sensitivity assay for Plasmodium falciparum based on a highly sensitive double-site lactate dehydrogenase antigen-capture enzyme-linked immunosorbent assay. Am J Trop Med Hyg 64(5-6):233-41.

Duraisingh MT, Cowman AF. 2005. Contribution of the pfmdr1 gene to antimalarial drug-resistance. Acta Trop 94(3):181-90.

Duraisingh MT, Curtis J, Warhurst DC. 1998. Plasmodium falciparum: detection of polymorphisms in the dihydrofolate reductase and dihydropteroate synthetase genes by PCR and restriction digestion. Exp Parasitol 89(1):1-8.

Duraisingh MT, Jones P, Sambou I, von Seidlein L, Pinder M, Warhurst DC. 1999. Inoculum effect leads to overestimation of in vitro resistance for artemisinin derivatives and standard antimalarials: a Gambian field study. Parasitology 119 (Pt 5):435-40.

Duraisingh MT, Jones P, Sambou I, von Seidlein L, Pinder M, Warhurst DC. 2000a. The tyrosine-86 allele of the pfmdr1 gene of Plasmodium falciparum is associated with increased sensitivity to the anti-malarials mefloquine and artemisinin. Mol Biochem Parasitol 108(1):13-23.

Duraisingh MT, von Seidlein LV, Jepson A, Jones P, Sambou I, Pinder M, Warhurst DC. 2000b. Linkage disequilibrium between two chromosomally distinct loci associated

with increased resistance to chloroquine in Plasmodium falciparum. Parasitology 121 (Pt 1):1-7.
Durand R, Jafari S, Vauzelle J, Delabre JF, Jesic Z, Le Bras J. 2001. Analysis of pfcrt point mutations and chloroquine susceptibility in isolates of Plasmodium falciparum. Mol Biochem Parasitol 114(1):95-102.
Echeverry DF, Holmgren G, Murillo C, Higuita JC, Bjorkman A, Gil JP, Osorio L. 2007. Short report: polymorphisms in the pfcrt and pfmdr1 genes of Plasmodium falciparum and in vitro susceptibility to amodiaquine and desethylamodiaquine. Am J Trop Med Hyg 77(6):1034-8.
Eckstein-Ludwig U, Webb RJ, Van Goethem ID, East JM, Lee AG, Kimura M, O'Neill PM, Bray PG, Ward SA, Krishna S. 2003. Artemisinins target the SERCA of Plasmodium falciparum. Nature 424(6951):957-61.
Elabbadi N, Ancelin ML, Vial HJ. 1992. Use of radioactive ethanolamine incorporation into phospholipids to assess in vitro antimalarial activity by the semiautomated microdilution technique. Antimicrob Agents Chemother 36(1):50-5.
Farnert A, Bjorkman A. 2005. Limited advantage of multiple consecutive samples for genotyping Plasmodium falciparum populations during the first days of treatment. Am J Trop Med Hyg 73(1):204-6.
Ferdig MT, Cooper RA, Mu J, Deng B, Joy DA, Su XZ, Wellems TE. 2004. Dissecting the loci of low-level quinine resistance in malaria parasites. Mol Microbiol 52(4):985-97.
Ferreira ID, Lopes D, Martinelli A, Ferreira C, do Rosario VE, Cravo P. 2007. In vitro assessment of artesunate, artemether and amodiaquine susceptibility and molecular analysis of putative resistance-associated mutations of Plasmodium falciparum from Sao Tome and Principe. Trop Med Int Health 12(3):353-62.
Ferrer-Rodriguez I, Perez-Rosado J, Gervais GW, Peters W, Robinson BL, Serrano AE. 2004. Plasmodium yoelii: identification and partial characterization of an MDR1 gene in an artemisinin-resistant line. J Parasitol 90(1):152-60.
Foley M, Tilley L. 1998. Quinoline antimalarials: mechanisms of action and resistance and prospects for new agents. Pharmacol Ther 79(1):55-87.
Foote SJ, Thompson JK, Cowman AF, Kemp DJ. 1989. Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of P. falciparum. Cell 57(6):921-30.
Gorissen E, Ashruf G, Lamboo M, Bennebroek J, Gikunda S, Mbaruku G, Kager PA. 2000. In vivo efficacy study of amodiaquine and sulfadoxine/ pyrimethamine in Kibwezi, Kenya and Kigoma, Tanzania. Trop Med Int Health 5(6):459-63.
Greenwood BM, Bojang K, Whitty CJ, Targett GA. 2005. Malaria. Lancet 365(9469):1487-98.
Gregson A, Plowe CV. 2005. Mechanisms of resistance of malaria parasites to antifolates. Pharmacol Rev 57(1):117-45.
Happi CT, Gbotosho GO, Folarin OA, Bolaji OM, Sowunmi A, Kyle DE, Milhous W, Wirth DF, Oduola AM. 2006. Association between mutations in Plasmodium falciparum chloroquine resistance transporter and P. falciparum multidrug resistance 1 genes and in vivo amodiaquine resistance in P. falciparum malaria-infected children in Nigeria. Am J Trop Med Hyg 75(1):155-61.
Henry M, Diallo I, Bordes J, Ka S, Pradines B, Diatta B, M'Baye PS, Sane M, Thiam M, Gueye PM and others. 2006. Urban malaria in Dakar, Senegal: chemosusceptibility and genetic diversity of Plasmodium falciparum isolates. Am J Trop Med Hyg 75(1):146-51.

Holmgren G, Gil JP, Ferreira PM, Veiga MI, Obonyo CO, Bjorkman A. 2006. Amodiaquine resistant Plasmodium falciparum malaria in vivo is associated with selection of pfcrt 76T and pfmdr1 86Y. Infect Genet Evol 6(4):309-14.

Howard RJ, Uni S, Aikawa M, Aley SB, Leech JH, Lew AM, Wellems TE, Rener J, Taylor DW. 1986. Secretion of a malarial histidine-rich protein (Pf HRP II) from Plasmodium falciparum-infected erythrocytes. J Cell Biol 103(4):1269-77.

Jafari S, Le Bras J, Bouchaud O, Durand R. 2004. Plasmodium falciparum clonal population dynamics during malaria treatment. J Infect Dis 189(2):195-203.

Jambou R, Legrand E, Niang M, Khim N, Lim P, Volney B, Ekala MT, Bouchier C, Esterre P, Fandeur T and others. 2005. Resistance of Plasmodium falciparum field isolates to in-vitro artemether and point mutations of the SERCA-type PfATPase6. Lancet 366(9501):1960-3.

Johnson DJ, Fidock DA, Mungthin M, Lakshmanan V, Sidhu AB, Bray PG, Ward SA. 2004. Evidence for a central role for PfCRT in conferring Plasmodium falciparum resistance to diverse antimalarial agents. Mol Cell 15(6):867-77.

Kaddouri H, Djimde A, Dama S, Kodio A, Tekete M, Hubert V, Kone A, Maiga H, Yattara O, Fofana B and others. 2008. Baseline in vitro efficacy of ACT component drugs on Plasmodium falciparum clinical isolates from Mali. Int J Parasitol 38(7):791-8.

Kamya MR, Yeka A, Bukirwa H, Lugemwa M, Rwakimari JB, Staedke SG, Talisuna AO, Greenhouse B, Nosten F, Rosenthal PJ and others. 2007. Artemether-lumefantrine versus dihydroartemisinin-piperaquine for treatment of malaria: a randomized trial. PLoS Clin Trials 2(5):e20.

Khalil IF, Alifrangis M, Tarimo DS, Staalso T, Satti GM, Theander TG, Ronn AM, Bygbjerg IC. 2005. The roles of the pfcrt 76T and pfmdr1 86Y mutations, immunity and the initial level of parasitaemia, in predicting the outcome of chloroquine treatment in two areas with different transmission intensities. Ann Trop Med Parasitol 99(5):441-8.

Kosaisavee V, Suwanarusk R, Nosten F, Kyle DE, Barrends M, Jones J, Price R, Russell B, Lek-Uthai U. 2006. Plasmodium vivax: isotopic, PicoGreen, and microscopic assays for measuring chloroquine sensitivity in fresh and cryopreserved isolates. Exp Parasitol 114(1):34-9.

Krishna S, White NJ. 1996. Pharmacokinetics of quinine, chloroquine and amodiaquine. Clinical implications. Clin Pharmacokinet 30(4):263-99.

Laufer MK, Thesing PC, Eddington ND, Masonga R, Dzinjalamala FK, Takala SL, Taylor TE, Plowe CV. 2006. Return of chloroquine antimalarial efficacy in Malawi. N Engl J Med 355(19):1959-66.

Le Bras J, Deloron P, Ricour A, Andrieu B, Savel J, Coulaud JP. 1983. Plasmodium falciparum: drug sensitivity in vitro of isolates before and after adaptation to continuous culture. Exp Parasitol 56(1):9-14.

Makler MT, Hinrichs DJ. 1993. Measurement of the lactate dehydrogenase activity of Plasmodium falciparum as an assessment of parasitemia. Am J Trop Med Hyg 48(2):205-10.

Makler MT, Ries JM, Williams JA, Bancroft JE, Piper RC, Gibbins BL, Hinrichs DJ. 1993. Parasite lactate dehydrogenase as an assay for Plasmodium falciparum drug sensitivity. Am J Trop Med Hyg 48(6):739-41.

Martensson A, Ngasala B, Ursing J, Isabel Veiga M, Wiklund L, Membi C, Montgomery SM, Premji Z, Farnert A, Bjorkman A. 2007. Influence of consecutive-day blood sampling on polymerase chain reaction-adjusted parasitological cure rates in an antimalarial-drug trial conducted in Tanzania. J Infect Dis 195(4):597-601.

Martin RE, Kirk K. 2004. The malaria parasite's chloroquine resistance transporter is a member of the drug/metabolite transporter superfamily. Mol Biol Evol 21(10):1938-49.

Mayxay M, Barends M, Brockman A, Jaidee A, Nair S, Sudimack D, Pongvongsa T, Phompida S, Phetsouvanh R, Anderson T and others. 2007. In vitro antimalarial drug susceptibility and pfcrt mutation among fresh Plasmodium falciparum isolates from the Lao PDR (Laos). Am J Trop Med Hyg 76(2):245-50.

Mayxay M, Pukrittayakamee S, Chotivanich K, Looareesuwan S, White NJ. 2001. Persistence of Plasmodium falciparum HRP-2 in successfully treated acute falciparum malaria. Trans R Soc Trop Med Hyg 95(2):179-82.

Menard D, Djalle D, Manirakiza A, Yapou F, Siadoua V, Sana S, Matsika-Claquin MD, Nestor M, Talarmin A. 2005. Drug-resistant malaria in Bangui, Central African Republic: an in vitro assessment. Am J Trop Med Hyg 73(2):239-43.

Menard D, Yapou F, Manirakiza A, Djalle D, Matsika-Claquin MD, Talarmin A. 2006. Polymorphisms in pfcrt, pfmdr1, dhfr genes and in vitro responses to antimalarials in Plasmodium falciparum isolates from Bangui, Central African Republic. Am J Trop Med Hyg 75(3):381-7.

Mockenhaupt FP, Ehrhardt S, Eggelte TA, Agana-Nsiire P, Stollberg K, Mathieu A, Markert M, Otchwemah RN, Bienzle U. 2005. Chloroquine-treatment failure in northern Ghana: roles of pfcrt T76 and pfmdr1 Y86. Ann Trop Med Parasitol 99(8):723-32.

Moreno A, Cuzin-Ouattara N, Nebie I, Sanon S, Brasseur P, Druilhe P. 2001. Use of the DELI-microtest to determine the drug sensitivity of Plasmodium falciparum in Burkina Faso. Ann Trop Med Parasitol 95(3):309-12.

Mu J, Ferdig MT, Feng X, Joy DA, Duan J, Furuya T, Subramanian G, Aravind L, Cooper RA, Wootton JC and others. 2003. Multiple transporters associated with malaria parasite responses to chloroquine and quinine. Mol Microbiol 49(4):977-89.

Nguyen-Dinh P, Deloron PL, Barber AM, Collins WE. 1988. Plasmodium fragile: detection of a ring-infected erythrocyte surface antigen (RESA). Exp Parasitol 65(1):119-24.

Nkrumah LJ, Riegelhaupt PM, Moura P, Johnson DJ, Patel J, Hayton K, Ferdig MT, Wellems TE, Akabas MH, Fidock DA. 2009. Probing the multifactorial basis of Plasmodium falciparum quinine resistance: evidence for a strain-specific contribution of the sodium-proton exchanger PfNHE. Mol Biochem Parasitol 165(2):122-31.

Noedl H, Attlmayr B, Wernsdorfer WH, Kollaritsch H, Miller RS. 2004. A histidine-rich protein 2-based malaria drug sensitivity assay for field use. Am J Trop Med Hyg 71(6):711-4.

Noedl H, Bronnert J, Yingyuen K, Attlmayr B, Kollaritsch H, Fukuda M. 2005. Simple histidine-rich protein 2 double-site sandwich enzyme-linked immunosorbent assay for use in malaria drug sensitivity testing. Antimicrob Agents Chemother 49(8):3575-7.

Noedl H, Wernsdorfer WH, Miller RS, Wongsrichanalai C. 2002. Histidine-rich protein II: a novel approach to malaria drug sensitivity testing. Antimicrob Agents Chemother 46(6):1658-64.

Nsobya SL, Dokomajilar C, Joloba M, Dorsey G, Rosenthal PJ. 2007. Resistance-mediating Plasmodium falciparum pfcrt and pfmdr1 alleles after treatment with artesunate-amodiaquine in Uganda. Antimicrob Agents Chemother 51(8):3023-5.

Nzila-Mounda A, Mberu EK, Sibley CH, Plowe CV, Winstanley PA, Watkins WM. 1998. Kenyan Plasmodium falciparum field isolates: correlation between pyrimethamine and chlorcycloguanil activity in vitro and point mutations in the dihydrofolate reductase domain. Antimicrob Agents Chemother 42(1):164-9.

Ochong EO, van den Broek IV, Keus K, Nzila A. 2003. Short report: association between chloroquine and amodiaquine resistance and allelic variation in the Plasmodium

falciparum multiple drug resistance 1 gene and the chloroquine resistance transporter gene in isolates from the upper Nile in southern Sudan. Am J Trop Med Hyg 69(2):184-7.

Oduola AM, Omitowoju GO, Sowunmi A, Makler MT, Falade CO, Kyle DE, Fehintola FA, Ogundahunsi OA, Piper RC, Schuster BG and others. 1997. Plasmodium falciparum: evaluation of lactate dehydrogenase in monitoring therapeutic responses to standard antimalarial drugs in Nigeria. Exp Parasitol 87(3):283-9.

Olliaro P, Nevill C, LeBras J, Ringwald P, Mussano P, Garner P, Brasseur P. 1996. Systematic review of amodiaquine treatment in uncomplicated malaria. Lancet 348(9036):1196-201.

O'Neill PM, Bray PG, Hawley SR, Ward SA, Park BK. 1998. 4-Aminoquinolines--past, present, and future: a chemical perspective. Pharmacol Ther 77(1):29-58.

O'Neill PM, Posner GH. 2004. A medicinal chemistry perspective on artemisinin and related endoperoxides. J Med Chem 47(12):2945-64.

Parikh S, Ouedraogo JB, Goldstein JA, Rosenthal PJ, Kroetz DL. 2007. Amodiaquine metabolism is impaired by common polymorphisms in CYP2C8: implications for malaria treatment in Africa. Clin Pharmacol Ther 82(2):197-203.

Pettinelli F, Pettinelli ME, Eldin de Pecoulas P, Millet J, Michel D, Brasseur P, Druilhe P. 2004. Short report: High prevalence of multidrug-resistant Plasmodium falciparum malaria in the French territory of Mayotte. Am J Trop Med Hyg 70(6):635-7.

Pickard AL, Wongsrichanalai C, Purfield A, Kamwendo D, Emery K, Zalewski C, Kawamoto F, Miller RS, Meshnick SR. 2003. Resistance to antimalarials in Southeast Asia and genetic polymorphisms in pfmdr1. Antimicrob Agents Chemother 47(8):2418-23.

Plowe CV, Wellems TE. 1995. Molecular approaches to the spreading problem of drug resistant malaria. Adv Exp Med Biol 390:197-209.

Pradines B, Hovette P, Fusai T, Atanda HL, Baret E, Cheval P, Mosnier J, Callec A, Cren J, Amalvict R and others. 2006. Prevalence of in vitro resistance to eleven standard or new antimalarial drugs among Plasmodium falciparum isolates from Pointe-Noire, Republic of the Congo. J Clin Microbiol 44(7):2404-8.

Pradines B, Mabika Mamfoumbi M, Parzy D, Owono Medang M, Lebeau C, Mourou Mbina JR, Doury JC, Kombila M. 1998. In vitro susceptibility of Gabonese wild isolates of Plasmodium falciparum to artemether, and comparison with chloroquine, quinine, halofantrine and amodiaquine. Parasitology 117 (Pt 6):541-5.

Pradines B, Mabika Mamfoumbi M, Parzy D, Owono Medang M, Lebeau C, Mourou Mbina JR, Doury JC, Kombila M. 1999. In vitro susceptibility of African isolates of Plasmodium falciparum from Gabon to pyronaridine. Am J Trop Med Hyg 60(1):105-8.

Price RN, Cassar C, Brockman A, Duraisingh M, van Vugt M, White NJ, Nosten F, Krishna S. 1999. The pfmdr1 gene is associated with a multidrug-resistant phenotype in Plasmodium falciparum from the western border of Thailand. Antimicrob Agents Chemother 43(12):2943-9.

Price RN, Uhlemann AC, Brockman A, McGready R, Ashley E, Phaipun L, Patel R, Laing K, Looareesuwan S, White NJ and others. 2004. Mefloquine resistance in Plasmodium falciparum and increased pfmdr1 gene copy number. Lancet 364(9432):438-47.

Price RN, Uhlemann AC, van Vugt M, Brockman A, Hutagalung R, Nair S, Nash D, Singhasivanon P, Anderson TJ, Krishna S and others. 2006. Molecular and pharmacological determinants of the therapeutic response to artemether-lumefantrine in multidrug-resistant Plasmodium falciparum malaria. Clin Infect Dis 42(11):1570-7.

Quashie NB, Duah NO, Abuaku B, Koram KA. 2007. The in-vitro susceptibilities of Ghanaian Plasmodium falciparum to antimalarial drugs. Ann Trop Med Parasitol 101(5):391-8.
Raj DK, Mu J, Jiang H, Kabat J, Singh S, Sullivan M, Fay MP, McCutchan TF, Su XZ. 2009. Disruption of a Plasmodium falciparum multidrug resistance-associated protein (PfMRP) alters its fitness and transport of antimalarial drugs and glutathione. J Biol Chem 284(12):7687-96.
Ramharter M, Noedl H, Thimasarn K, Wiedermann G, Wernsdorfer G, Wernsdorfer WH. 2002. In vitro activity of tafenoquine alone and in combination with artemisinin against Plasmodium falciparum. Am J Trop Med Hyg 67(1):39-43.
Rason MA, Andrianantenaina HB, Ariey F, Raveloson A, Domarle O, Randrianarivelojosia M. 2007. Prevalent pfmdr1 n86y mutant Plasmodium falciparum in Madagascar despite absence of pfcrt mutant strains. Am J Trop Med Hyg 76(6):1079-83.
Reed MB, Saliba KJ, Caruana SR, Kirk K, Cowman AF. 2000. Pgh1 modulates sensitivity and resistance to multiple antimalarials in Plasmodium falciparum. Nature 403(6772):906-9.
Richards WH, Maples BK. 1979. Studies on Plasmodium falciparum in continuous cultivation. I. The effect of chloroquine and pyrimethamine on parasite growth and viability. Ann Trop Med Parasitol 73(2):99-108.
Ringwald P, Bickii J, Basco LK. 1996. In vitro activity of antimalarials against clinical isolates of Plasmodium falciparum in Yaounde, Cameroon. Am J Trop Med Hyg 55(3):254-8.
Ringwald P, Bickii J, Basco LK. 1999. In vitro activity of dihydroartemisinin against clinical isolates of Plasmodium falciparum in Yaounde, Cameroon. Am J Trop Med Hyg 61(2):187-92.
Saito-Ito A, Akai Y, He S, Kimura M, Kawabata M. 2001. A rapid, simple and sensitive flow cytometric system for detection of Plasmodium falciparum. Parasitol Int 50(4):249-57.
Sarr O, Myrick A, Daily J, Diop BM, Dieng T, Ndir O, Sow PS, Mboup S, Wirth DF. 2005. In vivo and in vitro analysis of chloroquine resistance in Plasmodium falciparum isolates from Senegal. Parasitol Res 97(2):136-40.
Sherman IW. 1961. Molecular heterogeneity of lactic dehydrogenase in avian malaria (Plasmodium lophurae). J Exp Med 114:1049-62.
Sherman IW. 1979. Biochemistry of Plasmodium (malarial parasites). Microbiol Rev 43(4):453-95.
Sidhu AB, Verdier-Pinard D, Fidock DA. 2002. Chloroquine resistance in Plasmodium falciparum malaria parasites conferred by pfcrt mutations. Science 298(5591):210-3.
Sisowath C, Stromberg J, Martensson A, Msellem M, Obondo C, Bjorkman A, Gil JP. 2005. In vivo selection of Plasmodium falciparum pfmdr1 86N coding alleles by artemether-lumefantrine (Coartem). J Infect Dis 191(6):1014-7.
Smeijsters LJ, Nieuwenhuijs H, Hermsen RC, Dorrestein GM, Franssen FF, Overdulve JP. 1996. Antimalarial and toxic effects of the acyclic nucleoside phosphonate (S)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine in Plasmodium berghei-infected mice. Antimicrob Agents Chemother 40(7):1584-8.
Smilkstein M, Sriwilaijaroen N, Kelly JX, Wilairat P, Riscoe M. 2004. Simple and inexpensive fluorescence-based technique for high-throughput antimalarial drug screening. Antimicrob Agents Chemother 48(5):1803-6.
Staedke SG, Kamya MR, Dorsey G, Gasasira A, Ndeezi G, Charlebois ED, Rosenthal PJ. 2001. Amodiaquine, sulfadoxine/pyrimethamine, and combination therapy for

treatment of uncomplicated falciparum malaria in Kampala, Uganda: a randomised trial. Lancet 358(9279):368-74.
Staedke SG, Mpimbaza A, Kamya MR, Nzarubara BK, Dorsey G, Rosenthal PJ. 2004. Combination treatments for uncomplicated falciparum malaria in Kampala, Uganda: randomised clinical trial. Lancet 364(9449):1950-7.
Thomas SM, Ndir O, Dieng T, Mboup S, Wypij D, Maguire JH, Wirth DF. 2002. In vitro chloroquine susceptibility and PCR analysis of pfcrt and pfmdr1 polymorphisms in Plasmodium falciparum isolates from Senegal. Am J Trop Med Hyg 66(5):474-80.
Tinto H, Rwagacondo C, Karema C, Mupfasoni D, Vandoren W, Rusanganwa E, Erhart A, Van Overmeir C, Van Marck E, D'Alessandro U. 2006. In-vitro susceptibility of Plasmodium falciparum to monodesethylamodiaquine, dihydroartemisinin and quinine in an area of high chloroquine resistance in Rwanda. Trans R Soc Trop Med Hyg 100(6):509-14.
Trager W, Jensen JB. 1976. Human malaria parasites in continuous culture. Science 193(4254):673-5.
Tran CV, Saier MH, Jr. 2004. The principal chloroquine resistance protein of Plasmodium falciparum is a member of the drug/metabolite transporter superfamily. Microbiology 150(Pt 1):1-3.
Triglia T, Cowman AF. 1994. Primary structure and expression of the dihydropteroate synthetase gene of Plasmodium falciparum. Proc Natl Acad Sci U S A 91(15):7149-53.
Triglia T, Menting JG, Wilson C, Cowman AF. 1997. Mutations in dihydropteroate synthase are responsible for sulfone and sulfonamide resistance in Plasmodium falciparum. Proc Natl Acad Sci U S A 94(25):13944-9.
Valderramos SG, Fidock DA. 2006. Transporters involved in resistance to antimalarial drugs. Trends Pharmacol Sci 27(11):594-601.
van Vianen PH, Thaithong S, Reinders PP, van Engen A, van der Keur M, Tanke HJ, van der Kaay HJ, Mons B. 1990. Automated flow cytometric analysis of drug susceptibility of malaria parasites. Am J Trop Med Hyg 43(6):602-7.
Vieira PP, Ferreira MU, Alecrim MG, Alecrim WD, da Silva LH, Sihuincha MM, Joy DA, Mu J, Su XZ, Zalis MG. 2004. pfcrt Polymorphism and the spread of chloroquine resistance in Plasmodium falciparum populations across the Amazon Basin. J Infect Dis 190(2):417-24.
Vinayak S, Biswas S, Dev V, Kumar A, Ansari MA, Sharma YD. 2003. Prevalence of the K76T mutation in the pfcrt gene of Plasmodium falciparum among chloroquine responders in India. Acta Trop 87(2):287-93.
Voruganti L, Cortese L, Owyeumi L, Kotteda V, Cernovsky Z, Zirul S, Awad A. 2002. Switching from conventional to novel antipsychotic drugs: results of a prospective naturalistic study. Schizophr Res 57(2-3):201-8.
Wang P, Lee CS, Bayoumi R, Djimde A, Doumbo O, Swedberg G, Dao LD, Mshinda H, Tanner M, Watkins WM and others. 1997. Resistance to antifolates in Plasmodium falciparum monitored by sequence analysis of dihydropteroate synthetase and dihydrofolate reductase alleles in a large number of field samples of diverse origins. Mol Biochem Parasitol 89(2):161-77.
Warhurst DC. 2003. Polymorphism in the Plasmodium falciparum chloroquine-resistance transporter protein links verapamil enhancement of chloroquine sensitivity with the clinical efficacy of amodiaquine. Malar J 2:31.
Wilson CM, Serrano AE, Wasley A, Bogenschutz MP, Shankar AH, Wirth DF. 1989. Amplification of a gene related to mammalian mdr genes in drug-resistant Plasmodium falciparum. Science 244(4909):1184-6.

Wongsrichanalai C, Lin K, Pang LW, Faiz MA, Noedl H, Wimonwattrawatee T, Laoboonchai A, Kawamoto F. 2001. In vitro susceptibility of Plasmodium falciparum isolates from Myanmar to antimalarial drugs. Am J Trop Med Hyg 65(5):450-5.

Wongsrichanalai C, Pickard AL, Wernsdorfer WH, Meshnick SR. 2002. Epidemiology of drug-resistant malaria. Lancet Infect Dis 2(4):209-18.

Woodrow CJ, Haynes RK, Krishna S. 2005. Artemisinins. Postgrad Med J 81(952):71-8.

Yeka A, Banek K, Bakyaita N, Staedke SG, Kamya MR, Talisuna A, Kironde F, Nsobya SL, Kilian A, Slater M and others. 2005. Artemisinin versus nonartemisinin combination therapy for uncomplicated malaria: randomized clinical trials from four sites in Uganda. PLoS Med 2(7):e190.

Zhang Y, Konig I, Schirmer RH. 1988. Glutathione reductase-deficient erythrocytes as host cells of malarial parasites. Biochem Pharmacol 37(5):861-5.

Zongo I, Dorsey G, Rouamba N, Dokomajilar C, Sere Y, Rosenthal PJ, Ouedraogo JB. 2007a. Randomized comparison of amodiaquine plus sulfadoxine-pyrimethamine, artemether-lumefantrine, and dihydroartemisinin-piperaquine for the treatment of uncomplicated Plasmodium falciparum malaria in Burkina Faso. Clin Infect Dis 45(11):1453-61.

Zongo I, Dorsey G, Rouamba N, Tinto H, Dokomajilar C, Guiguemde RT, Rosenthal PJ, Ouedraogo JB. 2007b. Artemether-lumefantrine versus amodiaquine plus sulfadoxine-pyrimethamine for uncomplicated falciparum malaria in Burkina Faso: a randomised non-inferiority trial. Lancet 369(9560):491-8.

CQ

APPENDIX

Appendix A. Nsobya, SL, Dokomajilar, C, Joloba, M, Grant Dorsey, and Philip J. Rosenthal.(2007). Resistance-mediating Plasmodium falciparum pfcrt and pfmdr1 alleles after treatment with artesunate-amodiaquine in Uganda. Antimicrobial agents and chemotherapy, 51(8), 3023-5.

Appendix B.. Complexity of *Plasmodium falciparum* clinical samples from Uganda during short-term culture Samuel L. Nsobya, Moses Kiggundu, Moses Joloba, Grant Dorsey, and Philip J. Rosenthal. J Infect Dis. 2008 Sep 22

Appendic C. Nawaz F, **Nsobya SL**, Kiggundu M, Joloba M, Rosenthal PJ. Selection of parasites with diminished drug susceptibility by amodiaquine-containing antimalarial regimens in Uganda. The Journal of infectious diseases. 2009 Dec; 200(11):1650-7.

Appendic D. **Nsobya SL**, Kiggundu M, Nanyunja S, Joloba M, Greenhouse B, Rosenthal PJ. In Vitro Sensitivities of Plasmodium falciparum to Different Antimalarial Drugs in Uganda. Antimicrobial agents and chemotherapy. 2010 Mar;54(3):1200-6.