Prevalence of Polymorphisms in *Plasmodium falciparum* Parasites in Pregnant and Non-pregnant Women and Potential Resistance to Antimalarial Drugs in western Kenya

By

Brenda Makena Mugambi

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Centre for Biotechnology and Bioinformatics

University of Nairobi

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University for examination.

Name: Brenda Makena Mugambi

Signature: Brenda Makena Mugambi Digitally signed by Brenda Makena Mugambi Date: 2020.10.27 13:54:23 +03'00'

Date: 27th October 2020

I confirm that this thesis was developed by the candidate under my supervision.

Name: Dr Victor A. Mobegi

Department of Biochemistry & CEBIB, University of Nairobi

Signature: Digitally signed by Victor Mobegi Date: 2020.10.27 15:32:01 +03'00'

Date: 27.10.2020

Name: Dr George Obiero

Centre for Biotechnology and Bioinformatics

Signature: Digitally signed by George Obiero Date: 2020.10.27 15:51:49 +03'00'

Date: 27th October 2020

Name: Dr. Ben Andagalu

US Army Medical Research Directorate – Africa / Kenya

Signature: Digitally signed by ben.andagalu@usamru-k.org DN: cn=ben.andagalu@usamru-k.org Date: 2020.10.27 14:16:20 +03'00'

Date: 27OCT2020

Dedication

I dedicate this work to God for being my source of strength and to my family for being my support.

Acknowledgements

I acknowledge God almighty for His never ending guidance and giving me patience and strength to carry out this work through both the good times and the difficult moments. I also acknowledge and thank my family for their support. Also, I acknowledge and thank all the members of the Malaria Drugs Resistance lab at the United States Army Medical Research Directorate Africa-Kenya who hosted me for the entire period of my research and assisted me in all the steps of the work as well as my supervisors Dr. Victor Mobegi, Dr. George Obiero and Dr. Ben Andagalu. I am immensely grateful for all their contributions and help.

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List of Abbreviations and Acronyms

ACTs: Artemisinin-based combination therapies

AL: Artemether lumefantrine

CI: Clonally identical

DNA: Deoxyribonucleic acid

H_E: Heterozygosity

HIV: Human immunodeficiency virus

IBD: Identical by descent

IDC: Intraerythrocytic developmental cycle

IPTp-SP: Intermittent preventive treatment sulfadoxine-pyrimethamine

IRS: Indoor residual spraying

ITNs: Insecticide treated nets

IUGR: Intrauterine growth retardation

K13: *Plasmodium falciparum* Kelch 13 propeller gene

LBW: Low birth weight

LLITNs: Long-lasting insecticidal nets

PCR: Polymerase chain reaction

P. falciparum: *Plasmodium falciparum*

Pfcrt: *Plasmodium falciparum* chloroquine resistance transporter gene

Pfdhfr: *Plasmodium falciparum* dihydrofolate reductase gene

Pfdhps: *Plasmodium falciparum* dihydropteroate synthase gene

Pfmdr1: *Plasmodium falciparum* multidrug resistance 1 gene

Pfmrp1: *Plasmodium falciparum* multidrug resistance protein 1 gene

Pfnhe-1: *Plasmodium falciparum* Na^+/H^+ exchanger gene

RFU: Relative fluorescence units

RNA: Ribonucleic acid

rRNA: Ribosomal RNA

RTS,S (AS01): GSK's malaria vaccine candidate, Mosquirix^{TM}, that targets the circumsporozoite

SNPs: Single nucleotide polymorphisms

SP: Sulfadoxine pyrimethamine

SSR: Simple sequence repeats

STR: Short tandem repeats

WHO: World Health Organization

Abstract

Malaria infection in pregnancy results in maternal anaemia, placental accumulation of parasites, low birth weight infants together with maternal mortality owing to diminished maternal malaria immunity. In regions having high malaria transmission, majority of the population are presumed immune to malaria, with non-pregnant women showing quicker clearance of parasites in comparison to the pregnant. This observation suggests that the non-immune environment in pregnant women may select for parasite strains that are linked to artemisinin resistance. Our study focussed on genetic variation in *Plasmodium falciparum* parasites in pregnant versus non-pregnant women.

Blood samples were collected at hours 0, 8, 24 and at days 7 and 28 from 75 women positive for malaria comprising of 50 pregnant women in their second or third trimesters and 25 non-pregnant women, enrolled in an ACT efficacy study at Ahero in Kenya. Malaria diagnosis was done for all samples using PCR targeting 18S rRNA gene of *Plasmodium*. SNP genotyping for the K13, *Pfmdr1, Pfmrp1, Pfdhfr, Pfdhps* and *Pfcrt* genes was done to determine mutations in parasites with varying clearance using Sanger sequencing and MassARRAY. For MassARRAY SNP genotyping, the calls for the genotypes were made using SpectroTyper 4.0 software (Agena). The K13 Sanger sequencing reads assembly and mapping to the reference 3D7 genome was done using CLC Main Workbench software and DNA Baser. SNPs analysis was done using multiple sequence alignment by Clustal W in Bioedit.

Of the 75 women; 45 consisting pregnant 2nd trimester (n = 15), pregnant 3rd trimester (n = 12) and non-pregnant women (n = 18) had samples at all study time points merited to be included in the analysis. The 45 individuals comprised: Hour 0 = 100.0 %; Hour 8 = 84.4 %; Hour 24 = 26.7 %; Day 7 = 24.4 % and Day 28 = 4.4 % samples positive for *Plasmodium*. At hour 0, *P. falciparum* species were observed in 20.0 % of pregnant women in second trimester, 33.3 % of pregnant women in third trimester and 72.2 % of the non-pregnant women. *P. malariae* parasites were present in 8.3 % of pregnant women in third trimester and *P. ovale wallikeri* parasites in 13.3 % of pregnant women in second trimester, 58.3 % of third trimester pregnant women and 27.8 % of the non-pregnant women. At hour 8 of study, 28.6 %, 11.1 % and 62.5 % of the women in the second and third trimesters of pregnancy and the non-pregnant women respectfully had *P. falciparum* parasites. *P. ovale wallikeri* infections were observed in 28.6 % second trimester pregnant women, 22.2 % of the pregnant women in third trimester and 37.5 % of women in non-pregnant group. At hour 24 of the study, *P. falciparum* infections were only present in the non-pregnant women and constituted 100.0 % of the infections while *P. ovale wallikeri* infections were detected in 66.7 % of pregnant women in the second trimester and 100.0 % of pregnant women in third trimester. On study follow up day 7 parasites were present only in the second trimester pregnant women and they consisted of 10.0 % *P. ovale wallikeri* parasites. No parasites were present on follow up day 28 of the study. A total of 225 samples including all subsequent positives were carried on to MassARRAY and Sanger sequencing for SNPs genotyping. For lacking some study time points, we eliminated 30 participants samples from the study.

Pregnant women showed slower clearance of non-*falciparum* parasites after 24 hours of treatment with ACTs. 2nd trimester pregnant women had the highest cases (40.0 %) followed by the 3rd trimester (16.7 %) and non-pregnant (5.6 %) with the lowest number of samples having parasites at hour 24. Parasites were present even on follow up day 7 in 66.7 % of the pregnant

women in 2nd trimester. None of the study participants had parasites present on follow up day 28 of the study. K13 sequencing showed no nonsynonymous nor synonymous mutations in our Western Kenya samples. The mutations common in SEA like C580Y, I543T were not present in our Kenyan samples. Those common to Africa like A578S and S522C (Uganda) were also not present in our study samples. For the MassARRAY SNP genotyping, *Pfmdr1* gene had low frequency of mutations at codon 86 and showed similar frequencies of mutations and wild genotypes at codon 184 in the pregnant and non-pregnant women. *Pfdhfr* gene had high frequency of wild genotypes at codons 16, 22 and 164 while codons 59 and 108 had high number of mutants. These frequencies were distributed similarly between the two groups of pregnant and the non-pregnant women. *Pfdhps* gene showed similar trends in pregnant and also non-pregnant women with codons 436, 581 and 613 having high frequency of the wild type genotypes and codon 437 being majorly mutant. *Pfcrt* gene had similar trends in both study arms with all codons being mostly wild type genotype. The *Pfmrp1* gene was wild type in codons 191, 437 and 1390 in both study groups with codon 876 having both wild and mutant genotypes expressed similarly in both the pregnant and the non-pregnant women.

The pregnant women were found to have higher cases of non-*falciparum* infections compared to the non-pregnant women. This could be credited to their low immunity and/or the IPTp-SP prophylaxis which targets *falciparum* parasites.

Pregnant women did not carry parasites strains having mutations conferring resistance to artemisinin. The study did not find statistical differences between the pregnant and non-pregnant women groups to support suggestions that low immunity in pregnancy could be a source of resistant parasites strains or their reservoir. Use of ACTs for uncomplicated malaria in pregnant women was not seen to affect parasites genotypes probably since the loss of immunity is transient, relative to parasite lifecycle, hence is not a risk factor for development of resistance to ACTs. This also means IPTp-SP continues being effective as prophylaxis for malaria during pregnancy although its role in selection of species need to be considered.

Chapter 1: Introduction

1.1 Background to the study

Malaria results from intracellular parasites of the *Plasmodium* genus spread by bites of infected female *Anopheles* mosquitoes that are vectors for malaria. Human malaria is the resultant of five different *Plasmodium* species: *P. falciparum, P. malariae, P. vivax, P. ovale,* and *P. knowlesi*. *P. knowlesi* a *Plasmodium* species which commonly infects animals, infects humans occasionally. However, reports of human to mosquito human transmission of such zoonotic kinds of malaria are yet to be documented (World Health Organization 2020). Recent studies have uncovered two other human infectious species of *Plasmodium* namely: *P. cynomolgi*, a simian *Plasmodium* parasite, shown to infect tourists in South East Asia (Hartmeyer et al. 2019) and *P. simium,* which is a *P. vivax* like simian *Plasmodium* parasite that was, in Rio de Janeiro, linked to zoonotic malaria transmission (Brasil et al. 2017). Among the *Plasmodium* species, *P. falciparum* poses the greatest threat and is the most lethal. The others are associated with mainly febrile illness and rarely do they lead to severe illness (Pasvol 2006; Daneshvar et al. 2009).

Nearly half of the global populace, approximately 3.2 billion individuals, remain under the threat of malaria. Although this disease is avoidable and treatable, in 2017 there occurred about 219 million malaria instances globally, which was a rise of 2 million cases from 2016. Mortalities from malaria in 2017 were 435,000 which was almost similar to the 451,000 mortality cases in 2016 (World Health Organization 2018). The World Health Organization (WHO) African region bears highest portion of the universal malaria problem. 92 % worldwide malaria instances and 93 % malaria fatalities happened in Africa. In sub-Saharan African region, 80 % global malaria burden was attributed to fifteen nations (World Health Organization 2018). In Kenya, malaria persists as a main cause of illness and death with more than 70 % of individuals under threat of the infection. Regions near lake Victoria and coast bear the greatest risk, with children under 5 years and expectant women being more susceptible to malaria (Kenya National Bureau of Statistics 2015) due to low immunity. Globally, other groups at risk include mobile populations, travelers from malaria-free areas and non-immune migrants (World Health Organization 2018).

Following autopsy examinations in Mozambique, malaria was shown to be the cause of up to 10 % of the maternal deaths in the country (Menéndez et al. 2008), hence this suggested that in the

endemic areas, malaria could directly be responsible for nearly 25 % of the fatalities among mothers (Schantz-Dunn and Nour 2009). In 2017, children below the age of five were estimated to contribute to 266,000 (61 %) of the global malaria deaths (World Health Organization 2018). Low birth weight (LBW) was previously attributed to 75,000-200,000 of these cases (Desai et al. 2007; Steketee et al. 2001).

Anopheles arabiensis is the major Kenyan vector for malaria, with parasites of *P. falciparum* being causative agents for the majority of the infections (Okara et al. 2010). Malaria accounts for financial unproductivity in many endemic countries, decreasing the yearly financial progression in various areas by up to 1.5 % (Sachs and Malaney 2002). The statistics are because millions of individuals remain unable to access essential services necessary for aiding in preventing and treating malaria (World Health Organization 2017b).

1.4 Malaria in pregnancy

Malaria infections during pregnancy presents a huge concern for public health as well as a major contributor to maternal together with infant illness and deaths specially in the countries predominant to malaria (World Health Organization 2018).

Variability in susceptibility, carriage and progression of malaria disease in endemic areas has been described (McLean et al. 2015) and associated with acquired immunity (Chandrasiri et al. 2016), presenting differences in clinical presentation of the disease across ages and gender (Mutsigiri et al. 2017). Studies have shown that acquired malaria immunity is protective and complements drugs leading to rapid parasite elimination (Doolan, Dobaño, and Baird 2009). Among women, conception leads to diminished immunity in a precisely timed manner, reflecting an immune clock of pregnancy in women delivering at term predisposing them to malaria (Aghaeepour et al. 2017). Pregnant women are particularly susceptible to infections by malaria parasites with those from low malaria transmission settings having bigger threat of severe form of malaria from *P. falciparum*. Red blood cells infected with *P. falciparum* sequester in placenta and disrupt sharing of nutrients between foetus and mother triggering intrauterine growth retardation (IUGR) (McGready et al. 2012). Consequently, individuals contracting malaria during pregnancy experience other adverse effects such as: anaemia in mothers, parasites accumulating in placenta, prematurity resulting to low weight at birth (LBW), exposure of foetus

to parasites, hereditary infection, new-born deaths as well as maternal mortality (Desai et al. 2007; McGready et al. 2012; Steketee et al. 2001). However, regions having high transmission of malaria, malaria is typically asymptomatic in the pregnant women and linked to mild and general symptoms, even though it also has adverse effects on the foetus (World Health Organization 2015). The threat of mothers getting infected with malaria is greatest in their second trimester (Desai et al. 2007). Older age, multigravidity and pregnancy in the third trimester have shown association with decreased risk of parasitemia, while rainy seasons are associated with increased parasitemia (Clerk et al. 2009). Pregnant immigrants or visitors from regions of low or zero transmission for malaria as well as pregnant women having Human Immunodeficiency Virus (HIV) infections bear greater threat from malaria infections (Kenya Ministry of Public Health and Sanitation 2010).

Host versus malaria parasites interactions have been implicated in selection of parasites populations globally (Wellems, Hayton, and Fairhurst 2009). This interaction has led to varying strain circulation in South East Asia (SEA), South America (SA) versus Sub-Saharan Africa (SSA) (Singh and Sharma 2016). Though response to policy recommended drugs and emergence of resistance has been central to selection, pre-existing immunity appears to be central in defining the rate at which these evolutions progress and is responsible for faster occurrence of resistance in SEA than SSA (Fairhurst and Dondorp 2016). In line with the immunity associated parasite selection, pregnant women that have compromised immunity living in endemic regions that have high and stable infection and re-infection rate present a selection reservoir for parasites strains (Ebrahim 1996; Fried and Duffy 2017). Policies and efforts to eliminate malaria infections and transmission should therefore take women who have reached the reproductive age into consideration.

The WHO recommends for all pregnant women in their second and third trimesters of pregnancy and having *P. falciparum* uncomplicated malaria be given artemisinin-based combination therapies to treat them (World Health Organization 2015). Pregnant women in their first trimester are not administered to ACTs and instead the WHO recommends treating uncomplicated *P. falciparum* malaria infections in this group using 7 days of quinine + clindamycin (World Health Organization 2015). The fast-acting and powerful component of artemisinin (i.e., dihydroartemisinin, artesunate and artemether) decreases the parasitemia

significantly in the initial three days of treating malaria. The partner drugs which have longer activity (i.e., mefloquine, lumefantrine, piperaquine or amodiaquine) then serve to eliminate the remaining parasites hence preventing the risk of recrudescent malaria infections. In addition to that, the longer-acting drug also gives post treatment prophylactic effects hence preventing development of new infections while the concentration of the drugs in the body exceeds the minimum concentration for inhibiting the parasites. Unfortunately lumefantrine has a shorter half-life for elimination giving a significantly shorter prophylactic effect after treatment with AL (Kloprogge et al. 2013), unlike when treated with other partner drugs like dihydroartemisinin (Tarning et al. 2012). Longer half-life of partner drugs is particularly of key importance in high-transmission regions for malaria in which a prolonged post-treatment prophylactic effect reduces frequency of acquiring new infections and consequently the rate of morbidity due to malaria. Therefore, the duration of the prophylactic action post treatment is a result of the strength as well as the drugs elimination half-life. Similar mode of action applies to the intermittent preventive therapy, like the IPTp-SP, whereby repetitive curing antimalarial doses get rid of impending asymptomatic malaria infection as well as preventing new malaria infections. However, ACTs are not presently endorsed during pregnancy for intermittent preventive therapy. Information about the pharmacokinetics, efficacy and safety of ACTs in pregnancy is limited, therefore evaluation studies are ongoing (World Health Organization 2015).

Currently, WHO endorses for all the pregnant women living in malaria endemic regions in Africa, as part of their antenatal care, to be administered to Intermittent preventive treatment with sulfadoxine-pyrimethamine (IPTp-SP) (World Health Organization 2015). As prophylaxis against malaria, in their second as well as third trimesters, expectant mothers and infants who live in high transmission regions in Africa are given Intermittent preventive therapy with sulfadoxine-pyrimethamine (IPTp-SP) (Menéndez et al. 2010). Regrettably, extensive drug resistance in numerous areas globally undermines the efficiency in addition to potency of Sulfadoxine-pyrimethamine. Intervention efforts using chemoprophylaxis such as IPTp-SP combined with insecticide-treated nets (ITNs) lowers parasitemia and reduces maternal anaemia (Eric B Fokam et al. 2016; Eric Bertrand Fokam et al. 2016). However, due to drug pressure, this approach could as well be involved in selection of resistant parasite strains. This study will investigate polymorphisms in genes involved in ACTs resistance as both the pregnant as well as non-pregnant women were treated uncomplicated malaria with AL. This study will also look at

mutations in *Pfdhps* and *Pfdhfr* genes linked to resistance in SP as a surveillance on efficacy of the IPTp-SP prophylaxis administered to the pregnant women.

1.5 The evolution of antimalarial drugs policy and use over the years

Chloroquine (CQ) served as first-line antimalarial medication and resistance in *Plasmodium falciparum* was documented between 1978 and 1988 in all countries in the tropics of Africa (Trape 2001). There came a policy change to use combination treatment of antimalarial drugs (Trape 2001) and Sulfadoxine-pyrimethamine (SP) then became the successor drug to CQ. In Kenya, SP was introduced for treating uncomplicated malaria infections as first line drug in 1998 (Okiro et al. 2010) replacing CQ which stood as preferred first line treatment (Shretta et al. 2000). SP resistance then resulted in the WHO recommending using artemisinin-based combination therapies (ACTs) in 2001 (World Health Organization 2001). In 2006, Artemether Lumefantrine (AL) come to be the first line drug replacement to SP in treating uncomplicated infections of malaria (Amin et al. 2007) and became availed broadly in both the public and private clinics, hospitals and health centers. Extensive failure to treatment due to parasites drug resistance constituted the main cause for replacement of SP and CQ which had been utilized widely being first line treatment for malaria infections that were not complicated (Ogutu et al. 2005; Okiro et al. 2010; Shretta et al. 2000).

1.6 Problem statement

The spread of malaria is contributed by anti-malarial drugs in addition to insecticides resistance, social and environmental changes (Greenwood et al. 2002). *P. falciparum* artemisinin resistance appeared in regions Southeast of Asia and today creates a risk in controlling malaria in other areas. Extended doses of artemisinin-based combination treatments (ACTs) are presently efficacious in regions where recommended 3-days regimen are not effective (Ashley et al. 2014). The persistent risk of mosquito and parasite forming resistance to insecticides and medicines creates a great threat to controlling and eliminating malaria as these are the backbone of the current interventions (Wanjala et al. 2015; Protopopoff et al. 2013). The hope for malaria elimination therefore, depends on research and the development of new and improved tools to combat malaria infections. Understanding the interaction between the parasites and human hosts among the pregnant together with the non-pregnant women has potential to inform policy on the continued use of ACTs to treating malaria in pregnant women if found to have an effect on the

response to treatment among the pregnant women in addition to development and carriage parasites strains bearing resistance among them too.

Artemisinin combination therapies (ACTs) resistance in Africa is yet to be fully documented and ACTs still continue being very effective first line drugs for treating *P. falciparum* malaria that is uncomplicated (Kamau et al. 2015; Ménard et al. 2016; Paloque et al. 2018; World Health Organization 2010a). My study targeted assessing possibility of potential resistance to ACTs in Africa, and in Kenya specifically. This was done through a surveillance study to analyse western Kenya samples for possible molecular markers for resistance to ACTs. Delayed parasites clearance is defined as clearance time > 2 days after ACTs administration. The parasites are still present on day 3 after ACTs treatment (Sowunmi et al. 2010; World Health Organization 2011). These parasites without fast clearance express genes that may be potential markers for ACTs resistance.

1.7 Justification

WHO identifies pregnant women as a population at increased risk of malaria (World Health Organization 2003). Malaria burden in the group is mainly due to their decreased immunity and is highest during the first and second trimester (Desai et al. 2007) as well as the first pregnancy but decreases in the third trimester and in multigravid instances (Clerk et al. 2009).

Not paying attention to their plight means they are presenting a semi-immune reservoir in an endemic region with high transmission rate that risks selecting for resistance and would disseminate to the general population. Describing the patterns of antimalarial drugs resistance expressed in *P. falciparum* parasites in the pregnant women in comparison to those in parasites from non-pregnant women who tend to clear parasites faster, will help understand better the role pregnant women may play in development and spreading of resistance to artemisinin in parasites.

1.8 Significance of study

Interactions between the hosts and malaria parasites have been associated with selection of parasites populations (Wellems et al. 2009). Pregnant women have compromised immunity and in endemic regions this confers susceptibility to malaria and makes them potential selection reservoirs for resistant parasite strains (Fried and Duffy 2017). WHO in 2012 recommended that pregnant women be given intermittent preventive treatment with sulfadoxine-pyrimethamine

(IPTp-SP) as prophylaxis (World Health Organization 2015). As the WHO continues to implement IPTp-SP it is essential to clarify the role of this regimen in selecting parasites population and the association of these strains with artemisinins resistance.

Artemisinin combination therapies (ACTs) are administered to pregnant women in 2nd and 3rd trimesters for malaria treatment (World Health Organization 2019). In Kenya artemether lumefantrine (AL) is the recommended ACT (Ministry of Public Health and sanitation & Ministry of Medical services 2010). In Africa, resistance to ACTs has however not yet been documented (World Health Organization 2010a). Objective of my study is determining variability in *Plasmodium falciparum* strains amongst the pregnant and the non-pregnant women in relation to artemisisnin resistance.

1.9 Hypothesis

1.9.1 That the non-immune system of the pregnant women could select for population of parasites which have association with artemisinin resistance based on their delay in clearance of the parasites after treatment.

1.9.2 That there is variation in polymorphisms of artemisinin resistance markers in *P. falciparum* parasites between pregnant and non-pregnant women owing to variances in their immunity.

1.10 Objectives

1.10.1 Main objective

To establish the prevalence of polymorphisms in *Plasmodium falciparum* parasites in pregnant and non-pregnant women and potential resistance to antimalarial drugs in western Kenya.

1.10.2 Specific objectives

1. To determine *Plasmodium* parasites carriage and the treatment outcomes of ACTs between pregnant and non-pregnant women from parasites clearance times.
2. To determine variants in *Pfmdr1*, *Pfmrp1*, K-13, *Pfcrt*, *Pfdhfr* and *Pfdhps* genes in pregnant and non-pregnant women that confer resistance to artemisinin.

Chapter 2: Literature review

2.1 Malaria symptoms and disease progression

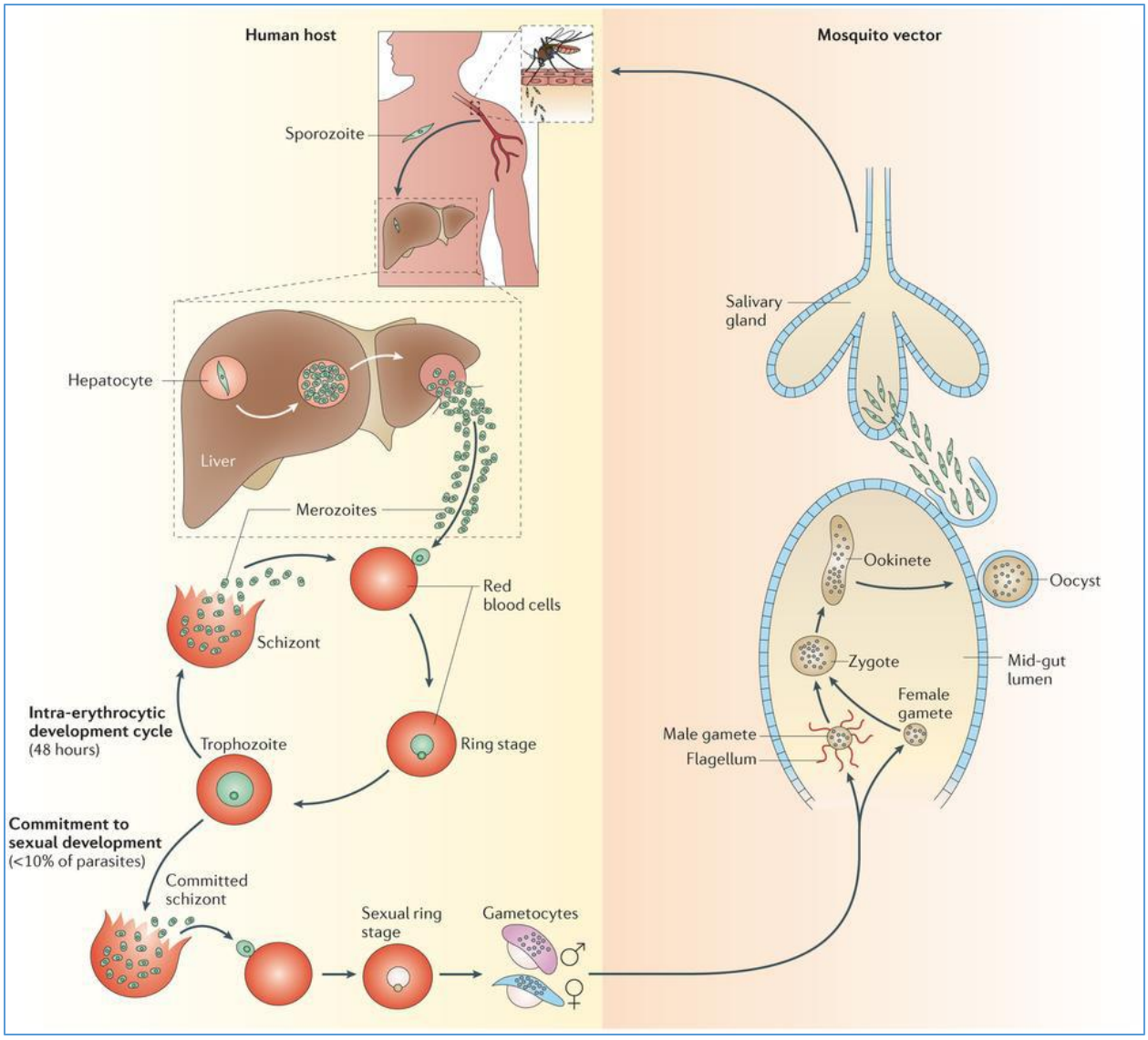
The primary malaria symptoms include: vomiting, headache, chills and fever and these ordinarily arise within 10 to 15 days following the bite of the mosquito. Without being treated promptly, malaria caused by *P. falciparum* quickly progresses to grave sickness and death. In countries having great proportions of transmission of malaria, young kids together with expectant women are especially susceptible to the serious results of being infected with malaria, which includes death. In areas of low endemicity for malaria, the disease's risk is distributed through all age clusters and relies on the extent of being exposed to bites of mosquitoes. It is in light with these cases that research in malaria is key especially in the developing countries (World Health Organization 2018).

2.2 Plasmodium falciparum life cycle

Plasmodium falciparum is an obligate endoparasite of the phylum apicomplexa. The parasite has a vestigial plastid, called apicoplast, which is an organelle involved in lipid biosynthesis and iron metabolism and occurs throughout the apicomplexan and is acquired through endosymbiosis (Keeling 2004). Plastids are homologous to chloroplasts in plants and algae. *P. falciparum* has two life stages: one in the humans as the hosts while another is in the mosquitoes as vectors (**Error! Reference source not found.**). Bites by an infected female *Anopheles* mosquito begins the phase of infection in human beings (Mazier et al. 1985; Soulard et al. 2015).

When taking blood meal, the mosquito injects sporozoites which migrate through blood stream to liver. In hepatocytes, sporozoites mature within 10-15 days to form schizonts which rapture to release merozoites. In *P. ovale* and *P. vivax*, some parasites in liver-stage (hypnozoites) enter an inactive state lasting months or years if the infection is untreated, initiating a new asexual reproduction cycle having clinical signs without fresh bite from mosquito, hence *P. vivax* malaria became commonly called malaria that relapses. Once hypnozoites mature, hepatocytes rapture releasing them into blood circulation. Merozoites thereafter rapidly enter mature erythrocytes and rapidly replicate asexually achieving high parasites burden and destroying the erythrocytes they infect, hence causing the clinical symptoms for malaria. In the intraerythrocytic developmental cycle (IDC), that lasts 48 hours, the merozoites upon invading the erythrocytes

first form rings stages of the parasites. The rings develop into trophozoites which then mature into schizonts that rapture releasing more merozoites in blood. These merozoites later attack new mature erythrocytes continuing the IDC. Some merozoites, however, differentiate into gametocytes in the bloodstream. The female gametocytes (macrogametocytes) and male gametocytes (microgametocytes) are then swallowed by pregnant female *Anopheles* mosquitoes when taking the blood meal hence initiating mosquitoes (sporogonic) phase of parasites life cycle. In vector mosquito stomach, microgametes penetrate the macrogametes to form zygotes. These zygotes then develop into the elongated motile ookinetes, which then penetrate through midgut walls to form oocysts. The oocysts then differentiate through meiosis into sporozoites which migrate into mosquitoes salivary glands with possibility of infecting humans when having blood meal, hence, initiating parasites cycle in human host. (Klein 2013; Malaria site 2018; CDC 2020; Medicines for Malaria Venture 2020)



Adapted from (Josling and Llinás 2015).

Figure 1: *Plasmodium falciparum* life cycle. Left half side shows the asexual blood phase within human hosts while right half side is the sexual stage in the mosquito vector.

2.3 Malaria treatment and resistance to drugs

Antimalarial drugs and insecticides resistance, in addition to, environmental and social changes are all contributors in the spreading and burden of malaria (Greenwood et al. 2002). There are four key methods to monitor antimalarial medicines effectiveness and resistance: curative

efficacy studies (gold standard), in vitro tests, using molecular markers as well as drug concentrations measurements. This study focussed on characterization of molecular markers of resistance which detect genetic changes linked to resistance. Several genes linked to *P. falciparum* antimalarials resistance have been identified. These are genes encoding: multidrug resistance protein 1 (*Pfmrp1*), multidrug resistance 1 gene (*Pfmdr1),* Na^+/H^+ exchanger (*Pfnhe-1),* dihydrofolate reductase (*Pfdhfr),* dihydropteroate synthase (*Pfdhps),* Kelch 13 propeller gene (K13), chloroquine resistance transporter (*Pfcrt)* and cytochrome b (World Health Organization 2010a).

Continuous rigorous monitoring of molecular markers linked to resistance of antimalarial drugs resistance is advised to enable timely resistance detection in the parasites. This will inform policies for treatment of malaria and enable quick and early efforts for containing and controlling new cases that might emerge. This is very important considering the distressing effects of both CQ and SP that were previously serving in the role of first-line malaria treatments in Kenya, as well as the similarities in epidemiology of malaria with regions in South East Asia where there have been reports of the development as well as spread of current ACTs resistance (Thriemer et al. 2014; World Health Organization 2017a; Dondorp et al. 2009).

2.4 Single Nucleotide Polymorphisms (SNPs)

Single nucleotide polymorphism (SNPs) are differences in DNA sequences which happen after one nucleotide – A, T, G or C – within a genome varies between individuals in a species or between paired chromosomes in a person.

2.5 The evolution of parasites resistance to antimalarial drugs

Spread of malaria is caused by anti-malarial drugs and insecticides resistance together with changes in society and the environment (Greenwood et al. 2002). Chloroquine (CQ) was broadly used in treating malaria as the first line drug and resistance in *P. falciparum* was reported between 1978 and 1988 in all countries of tropical Africa (Trape 2001). Molecular marker found to contribute to CQ resistance was a single mutation in the *Pfcrt* gene on chromosome 7 resulting in the amino acid change K76T (Fidock et al. 2000). Control of clinical symptoms has been proven to be better achieved when CQ is combined with sulfadoxine-pyrimethamine (SP) unlike using SP only and curing rate is higher with the regimen of triple combination (Le Bras 1999).

Looking at the documented evidence of *P. falciparum* malaria resistant to CQ, policy change to use combination therapy of antimalarials came highly recommended (Trape 2001). Sulfadoxine-pyrimethamine (SP) was then adapted in treating *P. falciparum* caused malaria as successor drug to CQ. Molecular markers linked to *P. falciparum* malaria resistance to SP were point mutations in *Pfdhfr* for pyrimethamine and *Pfdhps* for sulfadoxine, which are the main enzyme targets for antifolates in folate biosynthetic pathway(Gregson and Plowe 2005). Sequencing *Pfdhfr* and *Pfdhps* genes in strains showing different sensitivities towards antifolates brought to light proof of association between the two genes and resistance to antifolates (Bacon et al. 2009; Nzila et al. 2000; Sridaran et al. 2010). The different mutant forms of *Pfdhfr* contain S108N mutation, more mutations on codon 51 (N51I), codon 59 (C59R) as well as codon 164 (I164L) are found in isolates with higher resistance together with quadruple mutant S108N/ N51I/ C59R/ I164L that induces highest failure in SP treatment (Ecker, Lehane, and Fidock 2012). Mutations in *Pfdhps* linked to resistance to sulfadoxine are K540E, A613S/T, A581G, A437G, and S436A/F (Ecker, Lehane, and Fidock 2012).

However, due to emergence as well as the spreading resistance to SP, using artemisinin combination therapies (ACTs) was then proposed as a potentially longer lasting solution for antimalarial drug resistance crisis globally (Whitty et al. 2004).

The Chinese in 1970s discovered the antimalarial benefits of artemisinins as well as their products -Dihydroartemisinin (DHA), Artesunate (AS) and Artemether- but they began being widely used globally in 1990s. By then, the artemisinin monotherapy were broadly available in Asia and to preserve their potency the global drive to push for combination therapies began (Lin, Juliano, and Wongsrichanalai 2010; World Health Organization 2006a). In 2001, WHO endorsed use of Artemisinin-based combination therapy (ACTs), for treating *P. falciparum* uncomplicated malaria in countries where resistance to conventional antimalarials was observed due to its high efficacy and potential to resist spread of drug resistance (World Health Organization 2001, 2006b, 2006a).

Not only do artemisinins rapidly clear parasites that are asexual causing symptomatic infections in the blood-stage, they as well decrease the number of parasites in the sexual-stage (gametocytes) that are accountable for spreading the parasites (Lin, Juliano, and Wongsrichanalai 2010). Artemisinin combinations remain effective since artemisinin component

eliminates a large proportion of the parasites during the start of therapy while its partner drug which is eliminated at a slower rate clears the remaining parasites (Nicholas J White 2004). Implementation of ACTs as a replacement for the failed chloroquine and SP has reduced malaria-related sickness and death (Carrara et al. 2006).

In countries endemic to malaria, artemisinins currently constitute the core components of the first-line malaria treatments. *P. falciparum* resistance to artemisinin emergence in South East Asia creates great danger in controlling and eliminating malaria. It is usually detected as slow parasite clearance rate (Dondorp et al. 2009; Thriemer et al. 2014; World Health Organization 2017a). Clinical definition of resistance to artemisinins is characterized by reduction in parasites clearance rate in vivo (Dondorp et al. 2009) which is seen as an extended parasite clearance half-life (N. J. White 2011).

Artemisinin has been shown to induce a state of dormancy on the *P. falciparum* parasites that arrests growth of the parasites at ring stage after exposure to artemisinin drugs and later on causing recrudescence with the parasites recovering and resuming growth once the drug pressure is withdrawn. This may be a key factor in failure of ACTs treatment for malaria (Chotivanich et al. 2014; Paloque et al. 2018; Witkowski et al. 2013). Molecular markers linked to *P. falciparum* malaria artemisinins resistance are *Pfcrt, Pfmdr1* and the *P. falciparum* SERCA ortholog – PfATPase6, in addition to mutations observed in kelch-13 (K13) gene propeller region(Patel et al. 2017). The SERCA PfATPase6 mutations in *P. falciparum* have shown association with artemether resistance, a partner drug in ACTs (Jambou et al. 2005). Specific alleles in the *Pfcrt* and the *Pfmdr1* genes together with *Pfmdr1* copy numbers have been connected with a decrease in ACTs partner drugs lumefantrine and amodiaquine susceptibility (Malmberg et al. 2013). In South-East Asia, resistance to partner drug mefloquine has shown relation to *Pfmdr1* gene copy numbers increase (Price et al. 2004). A specific single nucleotide polymorphism, SNP (184F), in Cambodia is also linked to mefloquine resistance (Vinayak et al. 2010). *Pfmdr*1 gene variation in copy number is rarely observed in Africa as mefloquine is not commonly used (Dokomajilar et al. 2006). The SNP K76 in *Pfcrt* as well as mutations in *Pfmdr*1 are associated with decreased response of the parasites to lumefantrine, another partner drug of ACT in some sections of Africa together with South East Asia (Dokomajilar et al. 2006).

2.6 Dangers caused by ACTs resistance

In 2007, resistance to artemisinin was initially discovered in western Cambodia and has presently also proven to be present in Greater Mekong area and threat magnitude has not yet been quantified to determine the potential human and economic cost (Lubell, Y., Dondorp, A., Guérin, P.J., Drake, T., Meek, S., Ashley, E., Day, N.PJ, White, N..J., & White 2014). As a result of extensive artemisinin resistance, over 116,000 fatalities are estimated yearly. Treatment costs on failed medical cases and managing malaria that is severe exceeded the predicted 32 million US dollars annually. Excessive morbidity and mortality resulted in productivity losses valued at 385 million US dollars annually whereby failed ACTS continued being used initially for treating malaria (Lubell, Y., Dondorp, A., Guérin, P.J., Drake, T., Meek, S., Ashley, E., Day, N.PJ, White, N..J., & White 2014).

Artemisinin resistance emergence in sub-Saharan Africa is therefore going to endanger thousands of lives greatly affecting illness and death associated with malaria as well as cause an increase in medical and economic costs which are estimated to be hundreds of millions of US dollars annually (Lubell, Y., Dondorp, A., Guérin, P.J., Drake, T., Meek, S., Ashley, E., Day, N.PJ, White, N..J., & White 2014; Slater et al. 2016). To date however, resistance to artemisinin is yet to be reported in Africa while ACTs continue being very effective in treating uncomplicated *P. falciparum* malaria (Kamau et al. 2015; Ménard et al. 2016; Paloque et al. 2018; S. M. Taylor et al. 2015; World Health Organization 2010a).

2.7 Steps taken in controlling and slowing down ACTs resistance spread

Research work has been done on the kelch-13 gene which has been shown to be a target for the ACTs (Chhibber-Goel and Sharma 2019; Pasupureddy et al. 2019). In South-East Asia, the studies have identified independent artemisinins resistance emergence which has currently spread through South East Asia mainland. It stays mainly linked to mutations in portions of the gene in *Plasmodium falciparum* which codes the propeller domains of kelch (K13) (Tun et al. 2016) which remain confined in that region (Ménard et al. 2016; Takala-Harrison et al. 2015). Where typical 3 - days therapies fail, prolonged artemisinin–based combination therapies (ACTs) courses are proving effective (Ashley et al. 2014). Apart from the known mutations of the kelch 13 gene, alternative research works have demonstrated that single nucleotide polymorphisms

(SNPs) have been observed within phosphatidylinositol 4-kinase associated with one pathway involved in resistance to artemisinin (Cerqueira et al. 2017).

2.8 Malaria treatment and control

Control of malaria requires an integrated approach targeting both treatment and prevention. Strategies to control malaria transmission and infection have been scaled up recently. To block chain of transmitting malaria, insecticides are employed for controlling of vectors through indoor residual sprays (IRS) and mosquito nets treated with insecticides (ITNs) also used as additional measures for controlling spread of ACTs resistance (Arama and Troye-Blomberg 2014). The greatly preferred forms of ITNs for public health programs are long lasting insecticidal nets (LLINs) (Eric B Fokam et al. 2016). Chemoprophylaxis can be administered to travellers to suppress the blood stage of malaria infections and antimalarial drugs can also be used to prevent malaria infections. Artemether-lumefantrine (AL) and atovaquone-proguanil (AP) both treat uncomplicated malaria. As travellers having uncomplicated malaria recovered quicker when administered AL compared to when treated with AP, AL became the most frequently prescribed antimalarial chemoprophylaxis (Grynberg et al. 2015). Malaria poses a great health risk to pregnant women. About 50 million pregnant women live in areas endemic for malaria and the World Health Organization recommends using ITNs as well as intermittent preventive treatment using sulfadoxine-pyrimethamine (IPTp-SP) to be given to infants and expectant mothers who live in areas of increased transmission in Africa in their second in addition to the third trimesters (Eric Bertrand Fokam et al. 2016; Menéndez et al. 2010; World Health Organization 2006a).

Repetitive SP dosages combined alongside ITNs were observed to be efficient for reduction of malaria parasites and enhancing increasing haemoglobin levels in expectant women (Eric B Fokam et al. 2016). Timely diagnosis and prompt treatment with antimalarial medicines also prevents severe disease and death in pregnant women infected with malaria. Quinine and clindamycin is commended for use for seven days treatment of uncomplicated malaria in expectant women during their first trimester. Quinine monotherapy is to be used when clindamycin cannot be accessed. When this therapy is not successful, Artesunate should be used in combination with clindamycin for seven days (World Health Organization 2010b). The WHO recommended ACTs for treating uncomplicated malaria in second as well as third pregnancy

trimester (World Health Organization 2006a, 2010b). In Kenya, AL is recommended for treating uncomplicated malaria being first line drugs in second plus third pregnancy trimesters (Kenya Ministry of Public Health and Sanitation 2010). In 2018, RTS,S/AS01 (Mosquirix^{TM}) vaccine for malaria started being administered to children in Kenya, Ghana and Malawi in a WHO malaria vaccine pilot programme. The vaccine has about 40 % efficacy (PATH 2018) and has also been shown to target circumsporozoite parasites in *P.falciparum* and is effective in preventing symptomatic as well as severe malaria disease among infants and in children between the ages of 5-17 months (Neafsey et al. 2015).

2.9 Symptomatic malaria

The study participants were pregnant and non-pregnant women having symptomatic malaria that is not complicated who presented at the hospital for treatment. Symptomatic malaria is characterized by clinical features which include: severe anemia (hemoglobin [Hb] < 5g/dl); chest indrawing or deep breathing (respiratory distress as a marker of metabolic acidosis); fever (body temperature ≥ 37.5˚C); impaired consciousness (Blantyre coma score [BCS] that is less than 5; jaundice and hypoglycaemia (Marsh et al. 1995). Children having great asexual parasite concentrations and people with symptoms constitute the highest proportions of gametocyte positives and have the highest gametocyte densities (Koepfli et al. 2015).

In low endemic non-African countries like India, severe clinical malaria is often fatal as a result of limited studies on the management of life-threatening symptoms like multiple organ dysfunction which is a key reason of mortality among children (Tripathy et al. 2007). For children admitted to hospital from Malawi having clinical malaria, acidaemia was found to occur more among children having complicated (cerebral) malaria in comparison to those bearing uncomplicated clinical malaria and patients with acidaemia showed slower mean rate of respiration as well as greater incidences of abnormalities in respiratory rhythm (T. E. Taylor, Borgstein, and Molyneux 1993). Lactic acidosis was also found to be a main reason for dying amongst children with severe malaria in Gambia, with lactic acid levels in the blood of the fatal cases being almost twice as much as that in survivors during admission and falling only slightly or rising among the fatalities while dropping in survivors after treatment (Krishna et al. 1994).

The treatment of uncomplicated malaria by AL (Coartem) was found to be more effective than the previously recommended artesunate plus sulfadoxine-pyrimethamine (AS + SP) hence the change to AL to be first-line uncomplicated malaria treatment drug (Adeel et al. 2016; Nega et al. 2016; Warsame et al. 2017). Patients with uncomplicated malaria in Indonesia were found having antibodies that recognized a broader spectrum of *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) antigens compared with patients with severe malaria, indicating exposure to wide variety of PfEMP1s is linked to protection from severe malaria (Duffy et al. 2016).

Chapter 3: Methodology

3.1 Study sites and patients

The study was done on archived samples collected during a clinical trial study on ACTs efficacy in pregnancy at Ahero, in Kisumu. Kisumu is located in western Kenya along Lake Victoria's shores. The region is lowland area having holoendemic malaria (manuscript in preparation). The parent study population comprised of a total of 50 expectant women during their second or the third trimesters and 25 non-expectant women who have uncomplicated malaria presenting to antenatal as well as outpatient departments of Ahero Sub-district hospital and Ombeyi Health Centre in 2014. All study participants received treatment following the Kenya National Treatment Guidelines for malaria during pregnancy with Artemether/lumefantrine (Coartem®) tablets having 20 mg artemether plus 120 mg lumefantrine. The pregnant women as well took IPTp-SP as prophylaxis for malaria during their pregnancy.

3.2 Inclusion criteria

The original clinical trial study inclusion criteria was: pregnancy in second or third trimester or non-pregnant females aged 18 - 40 years; ability to give informed consent; fever present (Temp ≥ 37.5 °C) or fever history within past 2 days; asexual *P. falciparum* mono or admixed infections with *P. falciparum* as the predominant infection; primary parasitaemia with a range of 1000 to 200,000 vegetative parasite/µl of blood; absence of severe as well as complicated malaria; haemoglobin ≥ 8 g/dl and patient must have ability to ingest of study drugs orally.

3.3 Exclusion criteria

The exclusion criteria for the parent study was: patients with complicated and/or severe malaria (WHO, 2000 classification Appendix 2), comprising severe anaemia (Hb < 8 g/dl) and hyper-parasitaemia (>200,000 parasites/µl); vomiting; diarrhea; history of allergy to artemether or lumefantrine; known history of heart disease or arrhythmia; patients taking concomitant medication that may interfere with study endpoints and treatment with antimalarial drugs days before day zero of study.

3.4 Ethical clearance

Ethical clearance for parent study was gotten from Kenya Medical Research Institute and United States Army Medical Research Directorate Africa–Kenya. Written informed consents were acquired from participants before inclusion into study.

3.5 Blood sample collection and confirmation of malaria infection

During the clinical trial study venous blood was collected, processed and handled according to the Appropriate Technology in Health (PATH) guidelines (PATH 2005). 2ml of blood was collected at pre-determined time points at hours 0, 8 and 24, Additional sampling was done on days 7 and 28 for screening of resistance markers at the different time points during and after treatment. Since resistance development in *P. falciparum* is a gradual process (Woodrow and White 2017), the timelines for sampling were selected to screen for resistance markers at the beginning of treatment (hour 0) and within the first 24 hours of ACTs treatment as well as follow up days to determine the parasite clearance times and possible reappearance of parasites after treatment was completed, which would be an indication of possible resistance to treatment. Although sampling at more time points would give a much better comparison of the parasites clearance over time, the study objectives were answered with our chosen study time points. Confirmation of infection was done using duplicate thin and thick blood smears using 10 % Giemsa staining for microscopy as well as using rapid diagnostic tests (RDT). Samples found positive using both RDT and microscopy were then incorporated into the study. Blood from finger pricks were then taken using FTA filter papers (Whatman Inc., Bound Brook, New Jersey, USA) collecting 3 spots of blood having around 100 µl each for extraction of DNA for parasite genotyping using polymerase chain reaction (PCR).

3.6 Confirmation of *Plasmodium* DNA

Of the 75 participants initially enrolled in the study: arm 1 having 25 pregnant women on their second trimester, arm 2 having 25 pregnant women on their third trimester and arm 3 having 25 non-pregnant women; 11 were excluded due to samples missing at given time points. 19 more participants had samples that tested negative for *Plasmodium* at the baseline day 0, after carrying out the very sensitive quantitative reverse transcriptase real- time PCR assay specific to the genus to detect and quantify *Plasmodium* through amplification of the 18S rRNA genes in

Plasmodium were subsequently eliminated from study. Therefore, 30 participants that did not fit study inclusion standards were eliminated; of which, 10 participants were from arm 1, 13 participants from arm 2 and 7 were participants from the third study arm.

Parasites genomic DNA extraction and purification from filter papers of FTA (Tun et al. 2016) was performed by means of QIAgen Blood DNA Mini kit; Qiagen protocol (Qiagen, Valencia), using manufacturer's instructions (QIAGEN 2016). To confirm presence of parasite DNA a very sensitive quantitative reverse transcriptase real- time PCR test specific to the genus was done to detect and quantify *Plasmodium* through amplification of 18S rRNA genes DNA (Kamau et al. 2011). The real-time PCR assay was done using Applied Bio system QuantStudio 6 flex (QuantStudio^{Tm} real-time PCR applied Biosystems by Thermos Fisher Scientific). Cycling settings were initial heating to 96 °C for 5 minutes. Then denaturing at 96 °C for 10 seconds and primers annealed at 60 °C for 30 seconds repeated for 40 cycles. The forward primer sequence was 5'-GCTCTTTCTTGATTTCTTGGATG-3' and reverse primer sequence was 5'-AGCAGGTTAAGATCTCGTTCG-3'. Probe sequence was 5'-ATGGCCGTTTTTAGTTCGTG-3' and it was labelled with 5' FAM (6-carboxyfluorescein) as the reporter and 3' TAMRA (6-carboxytetramethyl-rhodamine) as the TaqMan quencher dyes. (Kamau et al. 2011). For *P. falciparum* infection detection, probes and primers specific for species were used, sequence for forward primer as 5'-ATTGCTTTTGAGAGGTTTTGTTACTTT-3' and reverse primer sequence being 5'-GCTGTAGTATTCAAACACAATGAACTCAA-3'. The sequence for probe was 5'-CATAACAGACGGGTAGTCAT-3' labelled using VIC as the reporter TaqMan dye. (Veron et al. 2009). This qPCR assay was preferred because it is quick, poses low contamination risks, and very specific, sensitive as well as quantitative with ability to detect low parasitemia in patients with submicroscopic infections and early-stage malaria (Kamau et al. 2011). Despite the PCR targeting *Plasmodium* mitochondrial cytochrome c oxidase III (*cox3*) gene (Isozumi et al. 2015) being faster and more efficient than the 18S rDNA approach (Kamau et al. 2011) and the *cytb* method (Putaporntip, Buppan, and Jongwutiwes 2011), the study assay choice was effective in answering the objectives.

3.7 Assessing *P. falciparum* resistance genes

3.7.1 Nested PCR for determining polymorphisms in the genes

Samples for the 75 participants collected at the baseline time (day zero hour zero), hour 8 and reappearance of the parasites at hour 24 as well as time points collections on follow up days 7 and 28 were used in this study for analysis.

To determine genetic variants, polymorphisms in the genes *Pfmdr1* codons 86, 184, 1034, 1042 and 1246; *Pfmrp1* codons 191, 437, 876 and 1390, K-13, *Pfcrt* codons 72, 76, 271, 326, 356, 356_2 and 371; *Pfdhfr* codons 16, 22, 59, 108 and 164 together with *Pfdhps* codons 436, 437, 581 and 613 were evaluated by nested PCR then proceeded to directly sequence the K-13 gene using Sanger Sequencing method.

3.7.2 MassARRAY assay

The 96 samples that were positive for *Plasmodium* at the different time points were further analysed for presence of mutations (SNPs) using MassARRAY genotyping. *Pfdhps, Pfmrp*1*, Pfcrt, Pfdhfr* and *Pfmdr*1 genes polymorphisms were assessed using nested PCR to determine genetic variants and frequency of the mutations.

Extension of single bases based on PCR on Sequenom MassARRAY platform (Agena bio-sciences, San Diego, CA, USA) was used for analysing the *Pfmdr1, Pfmrp1, Pfcrt, Pfdhfr* and *Pfdhps* genes following manufacturer's recommendation. The codons description was either being wild type, mutants or being mixed (comprising of the wild type together with the mutant genotypes existing in a single sample).

The 30 SNPs spread through all the target genes in *Plasmodium falciparum* were genotyped and analysed to identify variations in the sequences that may be responsible for the different responses of the parasites to antimalarial drugs. SNPs analysis determined whether parasite genotype influenced parasites clearance. They were: the *Pfmdr1* gene SNP N86Y on codon 86, SNP 184F on codon 184, SNP S1034C on codon 1034, SNP N1042D on codon 1042 and SNP D1246Y on codon 1246. Then the A16V SNP on codon 16, codon 22 mutations, the C59R SNP on codon 59, S108N mutation on codon 108 as well as the I164L SNP on codon 164 of the *Pfdhfr* gene. For the *Pfdhps* gene they included the: S436A/F SNP on codon 436, the A437G mutation on codon 437, the A437G SNP on codon 437, A581G SNP on codon 581 and the

A613S/T mutation on codon 613. For the *Pfcrt* we analysed SNP C72S on codon 72, SNP K76T on codon 76, Q271E SNP on codon 271, N326S SNP on codon 326, SNP I356T on codon 356, the I356L SNP on codon 356 as well as the R371I SNP on codon 371. We then analysed the H191Y, the S437A, the I876V and the F1390I SNPs for *Pfmrp1* on codons 191, 437, 876 and 1390.

For the MassARRAY assay, the PCR mix comprised of 10X PCR buffer, $MgCL_2$, dNTP mix, forward and reverse primers (0.5 µM) (pooled together in groups and sequence details in appendix section), Taq DNA polymerase enzyme and dH_20. To each well 4 µl of mastermix and 2 µl of sample were added for initial PCR. The conditions for the MassARRAY first PCR cycle were initial heating to 95 °C for 2 minutes. Followed by denaturation at 95 °C for 30 seconds and annealing of the primers at 56 °C for 30 seconds then elongation at 72 °C for 1 minute repeated for 44 cycles. Then holding at 72 °C for 5 minutes and final hold at 10 °C infinitely. SAP clean-up was then done on the amplicons with thermocycler set at 37 °C for 40 minutes then 85 °C for 5 minutes and holding at 10 °C infinitely. SAP mix consisted of 10X SAP buffer, SAP enzyme and dH_20. On the primary PCR amplicons plate, to each well 2 µl of the SAP mix was added. The second PCR is iPLEX PCR for single base extension. The iPLEX MIX contained 10X iPLEX buffer, iPLEX termination mix, adjusted primer mix (4 µM), iPLEX enzyme and dH_20. 2 µl of the iPLEX mix was added to each well. The cycling conditions were initial heating to 94 °C for 30 seconds, denaturation at 94 °C for 5 seconds then primer annealing at 52 °C for 5 seconds and elongation at 80 °C for 5 seconds, returning to step 3 five times then to step 2 40 times. Finally at 72 °C for 3 minutes and holding at 4 °C for infinity.

3.7.3 Purification of amplified products and Sanger sequencing of the K13 gene

For the K13 gene Sanger sequencing, K13 propeller region was first amplified. Master mix consisted of quantifast mix, forward and reverse primers (10 µM) with sequences K13_F 5′-TGG AAG ACA TCA GTC AAC CAG AGA-3′ and K13_R 5′-TTA TAT ATT TGC TAT TAA AAC GGA GTG-3′ and dH_20. To each well 23 µl of mastermix and 2 µl of sample were added. Cycling conditions for the K13 Sanger sequencing were initial heating lasting 5 minutes at 95 °C, then denaturing for 30 seconds at 95 °C, primers annealed for 45 seconds at 60 °C and elongation at 72 °C taking 3 minutes. The steps were repeated for 40 cycles then proceeded to 7 minutes at 72 °C and final holding for infinity at 4 °C. Afterwards, purification of the isolates

amplicons was done with Exosap-it® (Affymetrix, Santa Clara, CA) following protocol from manufacturer. 2 µl of exosap was added to each well with 10 µl of primary PCR amplicons and the cycling conditions set to 15 minutes at 37 °C, then 15 minutes at 80 °C and final hold temperature at 4 °C for infinity. Purified amplicons then went on to the Sanger sequencing step with the Big Dye PCR for single base extension using dDNTPs. To 2 µl of the cleaned amplicons 8 µl of the Big Dye mastermix was added to each well and constituted: 5X sequencing buffer, forward and reverse K13 gene primers (K13_2F 5′-GCC AAG CTG CCA TTC ATT TG-3′ and K13_3R 5′-GCC TTG TTG AAA GAA GCA GA-3′), big dye terminator and dH_20. The thermocycler conditions for this step were 95 °C going 5 minutes for initial heating, 95 °C taking 15 seconds for denaturation, 55 °C lasting 30 seconds to anneal primers, 68 °C - 2 minutes and 30 seconds for elongating then 68 °C for 3 minutes and ending holding at 4 °C for infinity. Sephadex cleaning using sephadex powder was then carried out on the secondary PCR amplicons before proceeding to the 3500 xL ABI Genetic analyser.

3.7.3.1 Agarose gel electrophoresis for confirmation of amplification of target gene

Following the amplification of the target regions, 2 % agarose gel was then used to separate the amplicons using electrophoresis following visualization and photographing of the gel by use of UVIsave gel documentation system (UVITEC 441228 Mini UVIsave-HD5 26M Gel Imager, 21 x 26 cm Transilluminator, 312 nm from UVITEC) to confirm amplification of the target gene. The target regions sequencing analysis was then carried out using 3500 xL ABI Genetic analyzer using version 3.1 of the big dye terminator method (Applied Biosystems, Foster City, CA).

3.8 Data analysis

Mass Array SpectroTyper 4.0 software (Sequenome) which translates mass differences converting to the nucleotide or SNPs variants was used to make genotype calls. For analyzing genetics influence on clearance, R software ggplot2 package was used to get the correlation between parasites expressed in pregnant and non-pregnant women. Mutations profiles for the K13 propeller gene were then carried out after sequencing of the genes by aligning against the reference 3D7 strain sequence.

Assembling contigs of the K13 propeller gene sequences that were generated and mapping to *Plasmodium falciparum* 3D7 reference genome retrieved from PlasmoDB (https://plasmodb.org/plasmo/app) (Accession number; PF3D7_1343700) was done using CLC

Main Workbench version 8.1 (QIAGEN, Aarhus, Denmark; available at https://digitalinsights.qiagen.com/) and DNA baser version 3x (https://www.dnabaser.com/). Multiple sequence assembly of the sequences was done using Clustal W embedded in BioEdit software version 7.2.5 (Hall 1999) with analysis of the SNPs done using the same BioEdit Sequence Alignment Editor.

Nucleotide sequences were translated into their protein sequences and analysed for presence of mutations found common in South East Asia like the C580Y, I 543T, Y493H, F446I, P574L, P553L, N458Y and R561H in our Kenyan samples. Those SNPs common to Africa like the A578S, V568G, D584Y, R539K, N585K, G665C, F583L, S522C (Uganda), V520A (West Central & East Africa), N531I (Ethiopia), V581F (Ghana), A676V, V534A and M579I were also assessed in the sequences from our Western Kenya samples. We also analysed the assembled sequences for the existence of any new mutations in our samples from Western Kenya.

Statistical analysis was done by use of CLC Main Workbench software version 8.1 (QIAGEN 2019) and R software version 3.6.1 (The R foundation 2019) to visualise the relationship between the *Plasmodium falciparum* isolated from the pregnant and non-pregnant women. Categorical data made up of genotype polymorphisms within the *Pfmdr1*, *Pfmrp1*, *Pfcrt*, *Pfdhfr* and the *Pfdhps* genes was then analysed as proportions displaying the frequency rates. Chi-square test was carried out to analyse the variations in point mutations frequencies in *Pfcrt*, *Pfdhps*, *Pfmdr1*, *Pfdhfr* and *Pfmrp1* genes between the pregnant and the non-pregnant women. To evaluate genes variations in parasites carried by the two study groups, polymorphism frequencies of the individual codons were compared. Additional comparisons of the frequencies of the genotypes were further done using Chi-square tests, to compare parasites genotypes between both groups. To get corrected *P* values of the tests, Yates' correction was done on the value of the Chi-square test. The statistical analysis was done using the 5 % level of significance in addition to corresponding 95 % Confidence Interval (CI).

Chapter 4: Results

4.1 Genus specific 18S rRNA PCR for diagnosis of *Plasmodium* infections

A total of 225 samples that met all study requirements were included in the subsequent assays of which 96 (42.7 %) were positive for *Plasmodium*. The first assay being the confirmation of the *Plasmodium* species present in all the samples. At baseline time day 0; arm 1 had 20.0 % (n = 3) of the samples being *P. falciparum* positive, 13.3 % (n = 2) *P. ovale wallikeri* positive and no samples having *P. malariae* or *P. ovale curtisi*. Arm 2 had 33.3 % (n = 4) being *P. falciparum* positive, 8.3 % (n = 1) positive for *P. malariae* and 58.3 % (n = 7) *P. ovale wallikeri* positive samples. Arm 3 had 72.2 % (n = 13) of the samples testing positive for *P. falciparum*, 27.8 % (n = 5) positive for *P. ovale wallikeri* with no *P. ovale curtisi* and *P. malariae* positive samples. At hour 8 of sampling; arm 1 had 28.6 % (n = 2) of the samples positive for *P. falciparum* and 28.6 % (n = 2) positive for *P. ovale wallikeri.* Arm 2 had 11.1 % (n = 1) *P. falciparum* positives and 22.2 % (n = 2) *P. ovale wallikeri* samples. Arm 3 had 62.5 % (n = 10) of the samples being positive for *P. falciparum* and 37.5 % (n = 6) *P. ovale wallikeri* positives. At hour 24; 0.0 % (n = 0) of samples in arm 1 as well as arm 2 were positive for *P. falciparum* whereas 100.0 % (n = 1) of arm 3 samples tested positive. Arm 1 had 66.7 % (n = 4) samples *P. ovale wallikeri* positive with arm 2 having 100 % (n = 2) *P. ovale wallikeri* positives. There were no samples positive for *P. malariae* and *P. ovale curtisi*. At follow up days 7 and 28; 0.0 % (n = 0) of samples on arms 1, 2 and 3 were positive for *P. malariae, P. falciparum* and *P. ovale curtisi. P. ovale wallikeri* was however present in 10 % (n = 1) of the samples in study arm 1 on follow up day 7. Table 1

Table 1: *Plasmodium* species diagnosis of each study arm according to time points.

	Arm 1					Arm 2					Arm 3				
	Plasmodium	***P. falciparum***	***P. malariae***	***P. ovale wallikeri***	***P. ovale curtisi***	***Plasmodium***	***P. falciparum***	***P. malariae***	***P. ovale wallikeri***	***P. ovale curtisi***	***Plasmodium***	***P. falciparum***	***P. malariae***	***P. ovale wallikeri***	***P. ovale curtisi***
Day 0	100.0	20.0	0.0	13.3	0.0	100.0	33.3	8.3	58.3	0.0	100.0	72.2	0.0	27.8	0.0
Hour 8	21.9	28.6	0.0	28.6	0.0	28.1	11.1	0.0	22.2	0.0	50.0	62.5	0.0	37.5	0.0
Hour 24	66.7	0.0	0.0	66.7	0.0	22.2	0.0	0.0	100.0	0.0	11.1	100.0	0.0	0.0	0.0
Day 7	100.0	0.0	0.0	10.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Day 28	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

4.2 Assessing frequency of mutations in *P. falciparum* genes

4.2.1 Mutations frequency on the *Pfmdr1, Pfmrp1, Pfcrt, Pfdhfr* and *Pfdhps* genes using MassARRAY SNP genotyping

Tables 2-6 show the frequency of the *Pfmdr1*, *Pfmrp1*, *Pfcrt*, *Pfdhfr* and *Pfdhps* SNPs in non-pregnant and pregnant women. SNPs are grouped as either wild type, mutants or mixed genotypes. Mixed genotypes are composed of both the wild and the mutant genotypes.

4.2.1.1 Frequency of mutations in the Pfmdr1 genes between pregnant and non-pregnant women

The *Pfmdr1* 86 locus had 92.7 % of pregnant women having wild type genotype whilst 78.8 % of the non-pregnant women were wild type at the locus. Mutant genotypes were 2.4 % in the pregnant group and 12.1 % among the non-pregnant. Mixed genotypes were present in both groups with the pregnant having 4.9 % compared to the 9.1 % in the non-pregnant women. **Error! Reference source not found.Error! Reference source not found.Error! Reference source not found.**In *Pfmdr1* 184 codon, 45.7 % of pregnant women had wild type alleles whereas 40.6 % of the non-pregnant women had the wild type genotype. Mutant genotypes were present in 47.8 % proportion of the pregnant women in addition to 56.3 % of the non-pregnant women. Mixed genotypes were also present in both groups and were 6.5 % among those pregnant and 3.1 % of the non-pregnant.

For the *Pfmdr1* 1034 locus 98.1 % of the pregnant women were wild type genotype whereas all (100.0 %) the non-pregnant women were wild type. There were no mutant alleles in the pregnant nor non-pregnant women observed. 1.9 % of the pregnant women had mixed genotypes while no one in the non-pregnant group had mixed genotypes. The *Pfmdr1* 1042 locus had no mutations nor mixed genotypes in pregnant and the non-pregnant groups. All had wild genotypes. The *Pfmdr1* 1246 locus had 97.7 % of the pregnant women being wild genotypes as well as 88.2 % amongst non-pregnant women. Mutations were present in 2.3 % of pregnant women coupled with 5.9 % of non-pregnant women. None of the women who were pregnant had mixed genotypes unlike 5.9 % of the non-pregnant ones. Table 2 below summarises this data.

Table 2: The frequency of mutations in the *Pfmdr*1 genes between pregnant and non-pregnant women.

			PREGNANT			NON-PREGNANT		
CODON POSITION	**SNP Position**	**3D7 reference aa (nucleotide)**	**Frequency %**			**Frequency %**		
			Wild	**Mutant**	**Mixed**	**Wild**	**Mutant**	**Mixed**
N86Y	258	N (A)	92.7	2.4	4.9	78.8	12.1	9.1
Y184F	552	Y (A)	45.7	47.8	6.5	40.6	56.3	3.1
S1034C	3102	S (A)	98.1	0.0	1.9	100.0	0.0	0.0
N1042D	3126	N (A)	100.0	0.0	0.0	100.0	0.0	0.0
D1246Y	3738	D (G)	97.7	2.3	0.0	88.2	5.9	5.9

4.2.1.2 Frequency of mutations on the Pfdhfr gene between pregnant and non-pregnant women

For *Pfdhfr* 16 locus 94.4 % of the pregnant women had wild genotypes while 5.6 % were mutants and none were mixed genotypes. The non-pregnant women were all wild types alleles with no mutations nor mixed genotypes present. The *Pfdhfr* 22 locus had 83.3 % pregnant women as wild types and 95.7 % non-pregnant women being wild types as well. 3.3 % of the

pregnant women had mutations whereas none of the non-pregnant women had mutations at this locus. Mixed genotypes were present in 13.3 % pregnant women plus 4.3 % non-pregnant women group.

In the *Pfdhfr* 59 locus, 14.6 % of the pregnant women were wild types as well as 6.3 % of the non-pregnant women. Mutant alleles were found in 77.1 % of pregnant women together with 90.6 % non-pregnant women. Mixed genotypes were present in 8.3 % of pregnant women and 3.1 % of non-pregnant. For *Pfdhfr* 108 locus, 2.1 % of the pregnant women were wild type while 97.9 % were mutants and no mixed genotypes were present. All the non-pregnant women were mutants at this locus. In the *Pfdhfr* 164 locus, wild genotypes are at 100 % in pregnant and the non-pregnant, whilst mutant and mixed are at 0 % in both groups. Table 3 below summarises the data.

Table 3: The frequency of mutations on the *Pfdhfr* gene between pregnant and non-pregnant women.

			PREGNANT			**NON-PREGNANT**		
CODON POSITION	**SNP Position**	**3D7 reference aa (nucleotide)**	**Frequency %**			**Frequency %**		
			Wild	**Mutant**	**Mixed**	**Wild**	**Mutant**	**Mixed**
A16V	48	A (C)	94.4	5.6	0.0	100.0	0.0	0.0
22	66	(G)	83.3	3.3	13.3	95.7	0.0	4.3
C59R	177	C (T)	14.6	77.1	8.3	6.3	90.6	3.1

S108N	324	S (G)	2.1	97.9	0.0	0.0	100.0	0.0
I164L	492	I (A)	100.0	0.0	0.0	100.0	0.0	0.0

4.2.1.3 Frequency of mutations on the Pfdhps gene between pregnant and non-pregnant women

Pfdhps 436 locus was wild type for all the pregnant together with the non-pregnant women samples. There were no mutants nor mixed alleles present in both groups. All the samples for pregnant and the non-pregnant women were mutants at the *Pfdhps* 437 locus. There were no wild types present in the samples nor any mixed genotypes. The *Pfdhps* 581 locus appeared wild type in all non-pregnant women and for 97.8 % of study pregnant women. Not any of non-pregnant women samples contained mixed genotypes at this locus while 2.2 % of the pregnant women were mixed genotypes. There were no mutant alleles present at this locus for pregnant together with non-pregnant women. In *Pfdhps* 613 locus, all the pregnant women were wild type genotypes with none of them having mutations or mixed genotypes. The non-pregnant women had 96.6 % of the samples being wild and 3.4 % being mutants at this locus. They had no mixed alleles present at the locus. The data is summarised in Table 4 below.

Table 4: The frequency of mutations on the *Pfdhps* gene between pregnant and non-pregnant women.

			PREGNANT			NON-PREGNANT		
CODON POSITION	**SNP Position**	**3D7 reference aa (nucleotide)**	**Frequency %**			**Frequency %**		
			Wild	**Mutant**	**Mixed**	**Wild**	**Mutant**	**Mixed**
S436A/F	1308	S (C)	100.0	0.0	0.0	100.0	0.0	0.0
A437G	1311	A(G)	0.0	100.0	0.0	0.0	100.0	0.0
A581G	1743	A (C)	97.8	2.2	0.0	100.0	0.0	0.0
A613S/T	1839	A (G)	100.0	0.0	0.0	96.6	3.4	0.0

4.2.1.4 Frequency of mutations on the Pfcrt gene between pregnant and non-pregnant women

All samples at *Pfcrt* 72 locus were wild types in pregnant and the non-pregnant women. Neither mutants nor mixed genotypes appeared from the two groups. *Pfcrt* 76 locus that is associated with tolerance of the parasites to lumefantrine had wild genotypes in 89.2 % of pregnant women and 74.2 % of non-pregnant women. Mutations appeared for 5.4 % of pregnant group and 16.1 % of non-pregnant women. There were also mixed genotypes present in 5.4 % of pregnant women plus 9.7 % of non-pregnant women. For the *Pfcrt* 271 locus, 93.3 % of pregnant women together with 78.8 % of non-pregnant women had wild genotypes. None of the pregnant women were

mutants while 6.1 % of the non-pregnant had mutant alleles at the locus. 6.7 % of the pregnant women were mixed genotypes together with 15.2 % of non-pregnant women. Samples were all wild type alleles at the *Pfcrt* 326 locus for both the pregnant and the non-pregnant women. No mutants nor mixed genotypes detected for both study groups. Pregnant women were all wild type genotype for the *Pfcrt* I356T mutations on codon 356 with none of the samples in the group being mutant nor mixed genotype. The non-pregnant had 94.3 % of their samples being wild type 5.7 % as mutant alleles but had no mixed genotypes. For the *Pfcrt* I356L SNPs on codon 356, all the study group samples were wild types. No mutants nor mixed genotypes were present in both pregnant and non-pregnant women. *Pfcrt* 371 locus constituted 73.8 % wild genotypes among the pregnant women, also 67.6 % of the non-pregnant women. Mutations presented in 9.5 % of pregnant women together with 2.9 % of non-pregnant group. Mixed alleles genotypes were in 16.7 % of pregnant women and 29.4 % of non-pregnant. Table 5 below gives a summary of the data.

Table 5: The frequency of mutations on the *Pfcrt* gene between pregnant and non-pregnant women.

			PREGNANT			NON-PREGNANT		
CODON POSITION	**SNP Position**	**3D7 reference aa (nucleotide)**	**Frequency %**			**Frequency %**		
			Wild	**Mutant**	**Mixed**	**Wild**	**Mutant**	**Mixed**
C72S	216	C (T)	100.0	0.0	0.0	100.0	0.0	0.0
K76T	228	K(A)	89.2	5.4	5.4	74.2	16.1	9.7
Q271E	813	Q(C)	93.3	0.0	6.7	78.8	6.1	15.2
N326S	978	N (A)	100.0	0.0	0.0	100.0	0.0	0.0
I356T	1068	I(A)	100.0	0.0	0.0	94.3	5.7	0.0
I356L	1068	I(T)	100.0	0.0	0.0	100.0	0.0	0.0
R371I	1113	R(G)	73.8	9.5	16.7	67.6	2.9	29.4

4.2.1.5 Frequency of mutations on the Pfmrp1 gene between pregnant and non-pregnant women

In the *Pfmrp1* gene, locus 191 had 75 % of the pregnant women as wild types and 25 % of them being mixed genotypes. All the non-pregnant women were wild type at the locus and no mixed genotypes were present in the group. No mutants were present in either the pregnant or the non-pregnant groups. The *Pfmrp1* 437 locus had wild type alleles in 94.4 % of pregnant women and 94.1 % of non-pregnant women. There were no mutant alleles in either groups. However, mixed genotypes were in 5.6 % and 5.9 % of pregnant as well as non-pregnant women respectively. The *Pfmrp1* 876 locus had wild types in 24.0 % of pregnant group plus 39.4 % of the non-pregnant. Mutant alleles appeared for 62.0 % of pregnant women as well as 27.3 % of non-pregnant women. In pregnant and non-pregnant group too, mixed alleles were 14.0 % and 33.3 % respectively. Majority of the samples in the two study groups were wild types at the *Pfmrp1* 1390 locus. They were 93.0 % among pregnant lot with 97.1 % among the non-pregnant. Mutants were not present in non-pregnant group but the pregnant women had 2.3 % mutations present. Both groups had mixed genotypes at 4.7 % for pregnant group of women and 2.9 % in non-pregnant. Table 6 below summarises the data.

Table 6: The frequency of mutations on the *Pfmrp*1 gene between pregnant and non-pregnant women.

			PREGNANT			NON-PREGNANT		
CODON POSITION	**SNP Position**	**3D7 reference aa (nucleotide)**	**Frequency %**			**Frequency %**		
			Wild	**Mutant**	**Mixed**	**Wild**	**Mutant**	**Mixed**
H191Y	573	H (C)	75.0	0.0	25.0	100.0	0.0	0.0
S437A	1311	S(T)	94.4	0.0	5.6	94.1	0.0	5.9
I876V	2628	I(A)	24.0	62.0	14.0	39.4	27.3	33.3
F1390I	4170	F (T)	93.0	2.3	4.7	97.1	0.0	2.9

4.2.2 Genotyping of the K13 propeller gene polymorphisms using Sanger sequencing

No mutations were present for K13 propeller gene after carrying out Sanger sequencing to identify SNPs in the study samples from western Kenya. After analysing SNPs that are common to South East Asia regions like the C580Y, I 543T, Y493H, F446I, P574L, P553L, N458Y and R561H, the study found them not present (Figure 2 and Figure 3). Also absent from the study samples were SNPs common to Africa like the A578S, V568G, D584Y, R539K, N585K, G665C, F583L, S522C (Uganda), V520A (West Central & East Africa), N531I (Ethiopia), V581F (Ghana), A676V, V534A and M579I (Figure 4 and Figure 5).

4.2.2.1 Screening for mutations on the K13 gene that are found in SEA (Figure 2 and Figure 3)

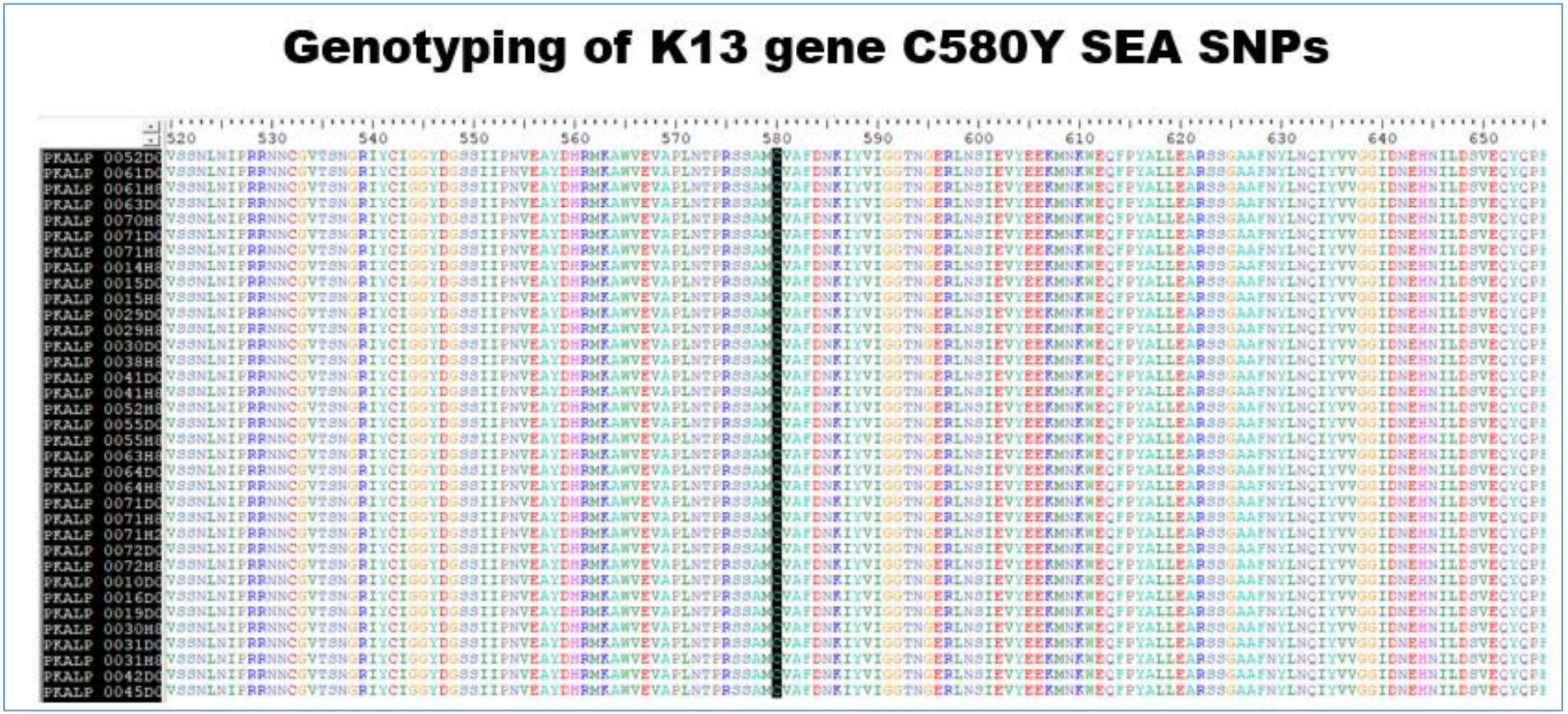


Figure 2: Genotyping of the K13 propeller gene C580Y SNPs common in SEA. At the top of the figure are the codon positions, the samples are listed on the left most pane of the figure (in black) and the nucleotide description for the different samples at the various codon positions are shown in the coloured letters. The SNPs common in SEA at codon 580 were not present in our samples from Western Kenya.

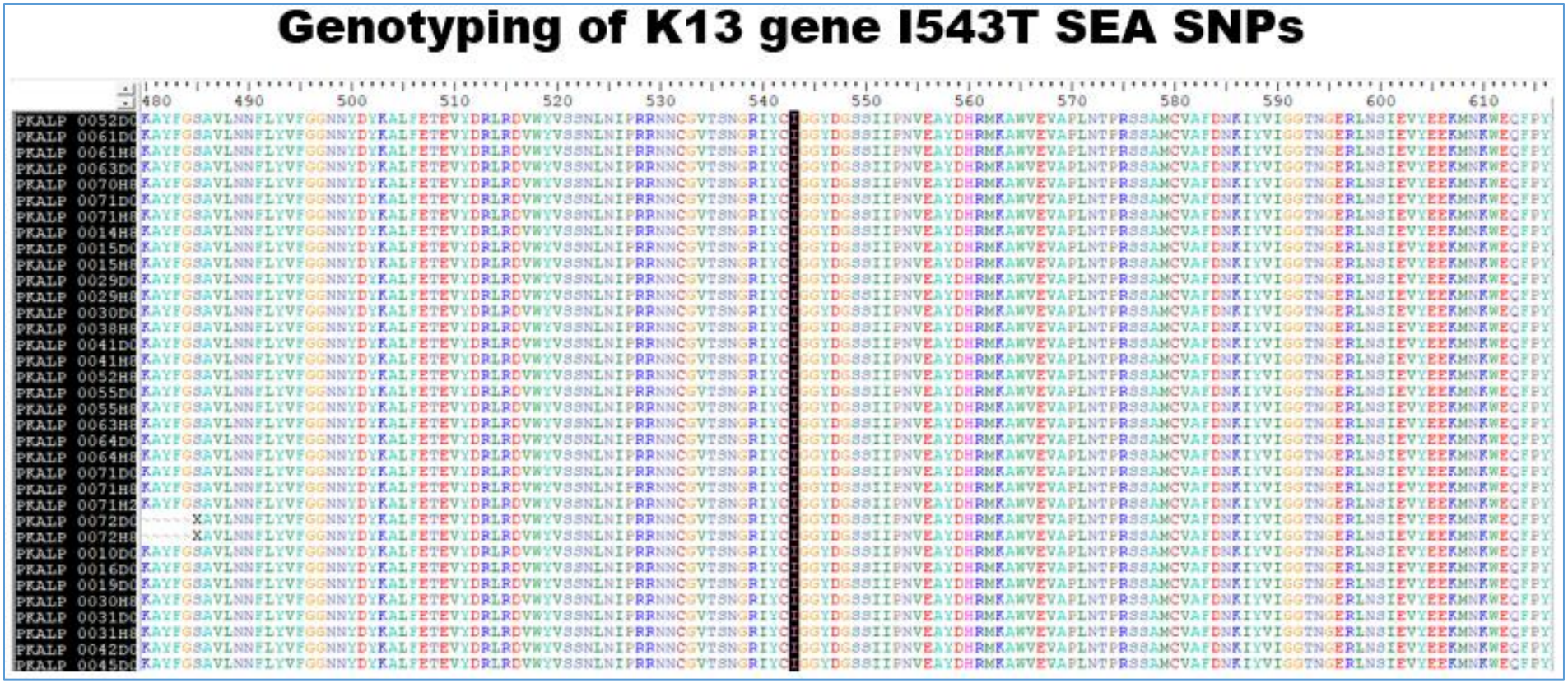


Figure 3: Genotyping of the K13 propeller gene I543T SNPs common in SEA. At the top of the figure are the codon positions, the samples are listed on the left most pane of the figure (in black) and the nucleotide description for the different samples at the various codon positions are shown in the coloured letters. The SNPs common in SEA at codon 543 were not present in our samples from Western Kenya.

4.2.2.2 Screening for mutations on the K13 gene that are found in Africa (Figure 4 and Figure 5)

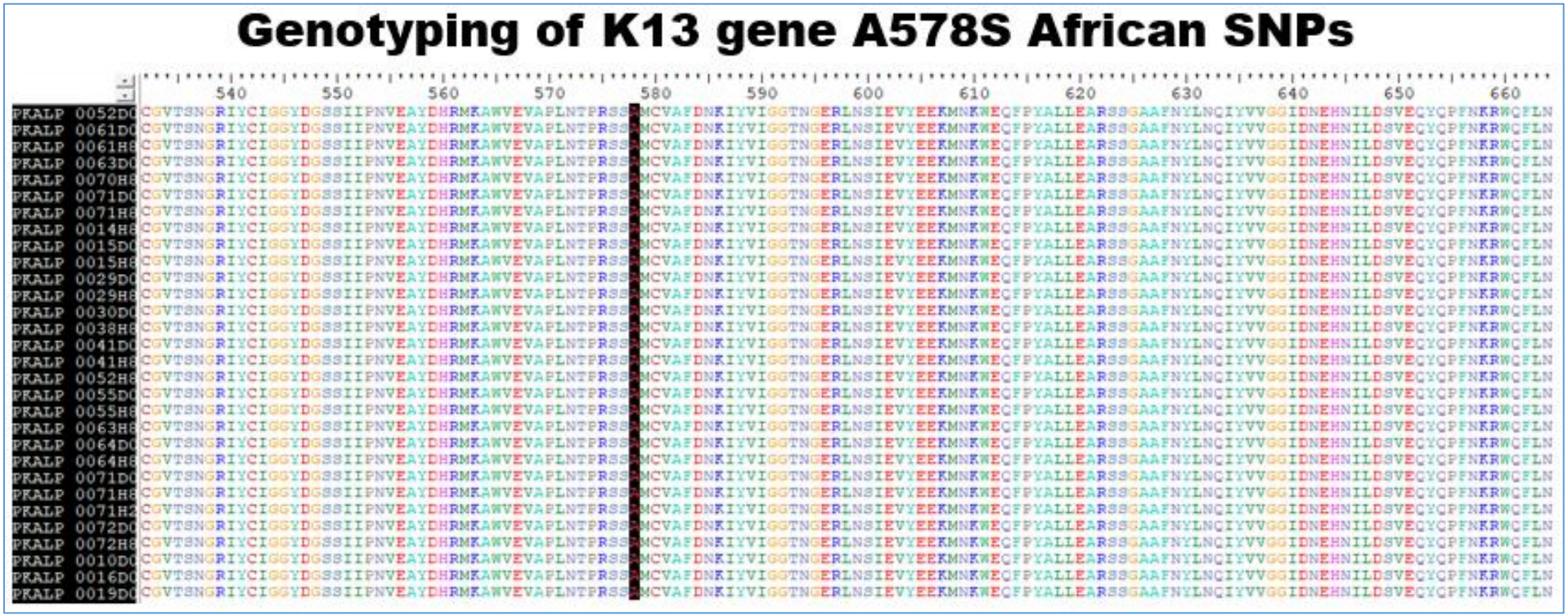


Figure 4: Genotyping of the K13 propeller gene A578S SNPs common in Africa. At the top of the figure are the codon positions, the samples are listed on the left most pane of the figure (in black) and the nucleotide description for the different samples at the various codon positions are shown in the coloured letters. The SNPs common in SEA at codon 578 were not present in our samples from Western Kenya.

Figure 5: Genotyping of the K13 propeller gene S522C SNPs common in Africa. At the top of the figure are the codon positions, the samples are listed on the left most pane of the figure (in black) and the nucleotide description for the different samples at the various codon positions are shown in the coloured letters. The SNPs common in SEA at codon 522 were not present in our samples from Western Kenya.

Chapter 5: Discussion

Selection of first line ACTs resistant malaria parasites is worrying to affected communities, medical specialists, investigators as well as specialists for controlling the development and spread of malaria infections. It poses a great public health worry that requires efficiency to address. To enhance the early and fast detecting of resistance emerging and spread, WHO endorses monitoring and assessing the drugs efficacy regularly using therapeutic efficacy studies and/or molecular resistance markers so as to advice policy change before the ACTs therapeutic efficacy falls below 90 % (World Health Organization 2010a).

We compared gene variations between pregnant and non-pregnant women to determine if pregnancy is a factor for resistance to ACTs and Sulfadoxine Pyrimethamine (SP). Confirmation of the *Plasmodium* infections was first done using qPCR and was followed by genotyping of the parasites between the pregnant as well as the non-pregnant women for identification of variations in parasites genes (SNPs). Genes of interest to our study were those involved in resistance in Sulfadoxine Pyrimethamine (SP) which are the *Pfdhfr* and the *Pfdhps* genes. Of more keen interest were those genes associated with resistance to ACTs which are *Pfmdr1, Pfmrp1* and the *Pfcrt* genes together with the K13 propeller gene. Total of 255 samples from study participants that met all the study requirements and had no samples missing in either of the five study time points were assayed and analysed to determine genetic variations between parasites strains expressed in the pregnant versus the non-pregnant women.

At day zero, 100.0 % (n = 45) of samples in all the study arms had *Plasmodium* of which in the second trimester pregnant women 20.0 % (n = 3) were *falciparum* and 13.3 % (n = 2) were *P. ovale wallikeri* parasites while for the third trimester pregnant women 33.3 % (n = 4) were *falciparum* infections, 8.3 % (n = 1) *P. malariae* and 58.3 % (n = 7) *P. ovale wallikeri* infections and 72.2 % (n = 13) of the samples in the non-pregnant women were *falciparum* infections and 27.8 % (n = 5) being *P. ovale wallikeri* infections. This showed a large number of the pregnant women had more non-*falciparum Plasmodium* infections compared to their non-pregnant counterparts who had majorly *falciparum* infections. This can be credited to the weakened immunity of pregnant women that predisposes them to mixed species infections. It could also be because of the SP in the prophylaxis given to the pregnant women that eliminates *falciparum*

parasites thereby resulting in the pregnant women group having low numbers of *falciparum* parasites infections compared to the non-*falciparum* parasites (Menéndez et al. 2010).

At hour eight, 21.9 % (n = 7) of the second trimester samples positive at day zero were positive for *Plasmodium* and 28.6 % (n = 2) of these were *falciparum* and 28.6 % (n = 2) *P. ovale wallikeri* infections. In the third trimester pregnant women 28.1 % (n = 9) of the samples positive at day zero had *Plasmodium* parasites at hour eight of which 11.1 % (n = 1) were *falciparum* and 22.2 % (n = 2) were *P. ovale wallikeri* infections. The non-pregnant women constituted 50.0 % (n = 16) of *Plasmodium* parasites infections of which 62.5 % (n = 10) were *falciparum* and 37.5 % (n = 6) were *P. ovale wallikeri* parasites at hour 8. This demonstrated that at hour eight of treatment with ACTs some parasites had started clearing from the body.

At hour 24, 66.7 % (n = 6) of the second trimester pregnant women had *Plasmodium* infections with 66.7 % (n = 4) of those being *P. ovale wallikeri* and none of them being *falciparum*. This was evidence that *falciparum* parasites cleared faster than the other *Plasmodium* species. This could be due to the activity of AL to target *falciparum* malaria parasites. The third trimester pregnancies had 22.2 % (n = 2) of their samples positive for *Plasmodium* with all being *P. ovale wallikeri* and none being *falciparum* infections as well. For the non-pregnant women, 11.1 % (n = 1) of them had *Plasmodium* at hour 24 and all were *falciparum* infections. The pregnant women had faster clearance of the *falciparum* parasites than the non-pregnant women however, they took longer in clearing the non-*falciparum* parasites. Still the non-pregnant women cleared all non-*falciparum* parasites by hour 24 of treatment with ACTs. This might be due to the weakened immunity of pregnant women making them more susceptible to reinfections by other *Plasmodium* parasites species. It could also be linked to the SP prophylaxis given to the pregnant women which targets the *falciparum* parasites (Menéndez et al. 2010).

During the follow up day 7 only the second trimester pregnant women had parasites present at 100.0 % (n = 10), with 10.0 % (n = 1) being *P. ovale wallikeri* infections while the rest had cleared the parasites. Second trimester women are the most at risk for malaria infections and have a weaker immune system compared to the third trimester women and the non-pregnant women (Desai et al. 2007). Therefore this could explain their delay in clearing the parasites after treatment with ACTs.

In addition to the weakened immunity of the pregnant women, they were also on prophylaxis for malaria. Since the pregnant women had received IPTp-SP as prophylaxis for malaria in pregnancy, the SP in their system may have served to eliminate the *falciparum* parasites, which are usually its target, hence they showed higher frequency of the non-*falciparum* parasites (Menéndez et al. 2010). The non-pregnant women however had higher *falciparum* infections at all the time points because they never received any treatment with SP to suppress the development of *falciparum* parasites infections.

Polymorphisms present in the K13, *Pfmdr1*, *Pfmrp1* and *Pfcrt* genes are highly linked to clinical failure in treatment with artemisinin (Dokomajilar et al. 2006; Malmberg et al. 2013). Polymorphisms in *Pfdhps* and *Pfdhfr* genes are connected with failure after treatment with SP (Bacon et al. 2009; Gregson and Plowe 2005; Nzila et al. 2000; Sridaran et al. 2010), with *Pfdhfr/Pfdhps* C59R, K540E, N51I, S108N/A437G quintuple mutant genotype being related to clinical SP failure during treatment (Ecker, Lehane, and Fidock 2012). The MassARRAY SNP analysis of the genes showed similar expression profiles of the wild types, mutants and mixed genotypes between pregnant and the non-pregnant women.

Chi-square test was employed to get variations in point mutations frequencies in *Pfmdr1*, *Pfdhps*, *Pfmrp1*, *Pfcrt* and, *Pfdhfr* genes between both pregnant as well as the non-pregnant participants. Polymorphism frequencies at individual codons were compared to evaluate variations in genes between pregnant group and non-pregnant. In comparison of parasites genotypes between pregnant women with the non-pregnant women, additional comparisons of frequencies of genotypes were further done using Chi-square tests. Yates' correction then done on the Chi-square value so as to get corrected *P* values of the tests. The statistical analyses then done at 5 % significant level with corresponding 95 % Confidence Interval (CI). No statistically significant variations were seen between pregnant and non-pregnant groups therefore showing that the pregnant women did not harbour resistance genes to ACTs treatment.

Sanger sequence analysis of K13 propeller gene to identify SNPs from our sample set from western Kenya showed no mutations were present. Analysis of the SNPs commonly found in South East Asia like the C580Y, I 543T, Y493H, F446I, P574L, P553L, N458Y and R561H were not present in our Western Kenya samples. Those SNPs common to Africa like the A578S, V568G, D584Y, R539K, N585K, G665C, F583L, S522C (Uganda), V520A (West Central &

East Africa), N531I (Ethiopia), V581F (Ghana), A676V, V534A and M579I were also not present in our samples. These results resembled those from another work by (Raman et al. 2019) from South Africa that found none of the 25 mutations linked to delay in clearance of parasites in South East Asia in their study samples after genotyping the K13 propeller gene.

Chapter 6: Conclusion and Recommendations

6.1 Conclusion

This study showed pregnant women did not carry parasites strains that have mutations which confer resistance to artemisinin. Despite their weakened immune system the pregnant women responded similar to the non-pregnant women when treated with ACTs. No variations having statistically significant differences in the two study groups that proved pregnancy to be a source for development of resistant parasites strains nor a reservoir for the resistant parasites that leads to their transmission and spread. Therefore, treating the pregnant women with ACTs just like the non-pregnant does not affect the parasites genotypes hence is not a threat factor for developing of resistance of parasites to ACTs. We also showed that SP is still effective as prophylaxis during pregnancy although its role in selection of non-*falciparum* parasites has to be analysed further.

6.2 Recommendations

Constant monitoring of molecular markers linked to antimalarial drugs resistance is recommended for enabling early detection of parasites resistance. This thus informs malaria treatment policies and enables quick and timely efforts for containing and controlling potential cases that may emerge. This is crucial considering the distressing effects of both CQ and SP that were previously serving as first-line malaria treatments in Kenya, as well as the similar malaria epidemiology with regions in South East Asia where development and spread of current ACTs resistance have been observed.

The study however had some shortcomings. First one was the small sample size which was limited by the original study. Although the size of the sample was small, it still answered my study objectives. The future studies however will benefit from having bigger sample sizes and freshly collected samples in order to have more concrete results and conclusions on the frequency of polymorphisms in *P. falciparum* parasites between pregnant and the non-pregnant women. We as well did not get the opportunity to analyse samples from all study time points, which could be helpful in giving better comparison of parasites clearance with time. Doing whole genome sequencing (WGS) of the samples is recommended for a much deeper analysis to

give a better comparison of parasites carried by the pregnant women group in comparison to those carried by the non-pregnant women.

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Appendices

QIAamp DNA protocol for purification of DNA from dried blood spots

Protocol: DNA Purification from Dried Blood Spots (QIAamp DNA Mini Kit)

This protocol is for purification of total (genomic, mitochondrial, and viral) DNA from blood, both untreated and treated with anticoagulants, which has been spotted and dried on filter paper (Schleicher and Schuell 903).

Important point before starting

- All centrifugation steps are carried out at room temperature (15–25°C).

Things to do before starting

- Prepare an 85°C water bath for use in step 2, a 56°C water bath for use in step 3, and a 70°C water bath for use in step 4.
- Equilibrate Buffer AE or distilled water to room temperature (15–25°C) for elution in step 10.
- Ensure that Buffer AW1 and Buffer AW2 have been prepared according to the instructions on page 16.
- If a precipitate has formed in Buffer AL or Buffer ATL, dissolve by incubating at 56°C.

Procedure

1. Place 3 punched-out circles from a dried blood spot into a 1.5 ml microcentrifuge tube and add 180 µl of Buffer ATL. Cut 3 mm (1/8 inch) diameter punches from a dried blood spot with a single-hole paper puncher.

2. Incubate at 85°C for 10 min. Briefly centrifuge to remove drops from inside the lid.

3. Add 20 µl proteinase K stock solution. Mix by vortexing, and incubate at 56°C for 1 h. Briefly centrifuge to remove drops from inside the lid.

Note: The addition of proteinase K is essential.

4. Add 200 µl Buffer AL to the sample. Mix thoroughly by vortexing, and incubate at 70°C for 10 min. Briefly centrifuge to remove drops from inside the lid. To ensure efficient lysis, it is essential that the sample and Buffer AL are mixed immediately and thoroughly.

Note: Do not add proteinase K directly to Buffer AL.

A white precipitate may form when Buffer AL is added to the sample. In most cases, the precipitate will dissolve during incubation. The precipitate does not interfere with the QIAamp procedure or with any subsequent application.

5. Add 200 µl ethanol (96–100 %) to the sample, and mix thoroughly by vortexing. Briefly centrifuge to remove drops from inside the lid.

It is essential that the sample and ethanol are mixed thoroughly.

6. Carefully apply the mixture from step 5 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.

Close each QIAamp Mini spin column to avoid aerosol formation during centrifugation.

7. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1 min.

Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.

8. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.

9. Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

This step helps to eliminate the chance of possible Buffer AW2 carryover.

10. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 150 µl Buffer AE or distilled water. Incubate at room temperature (15–25°C) for 1 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min.

Three punched-out circles (3 mm diameter) typically yield 150 ng and 75 ng of DNA from anticoagulated and untreated blood, respectively. If the yield from untreated blood is not sufficient, use 6 circles per prep instead of 3.

The volume of the DNA eluate used in a PCR assay should not exceed 10 %; for example, for a 50 µl PCR, add no more than 5 µl of eluate.

***Plasmodium* detection by PCR (plu assay)**

This procedure outlines the method for PCR for detection of *Plasmodium* species. The PLU assay is an *in vitro* diagnostic test for the rapid detection of *Plasmodium* spp. The test detects the presence of *Plasmodium* DNA in sample processed from whole blood or dried blood spots by the amplification of 18S rRNA gene sequence common to all species of *Plasmodium*.

All standard procedures in the lab for molecular analysis must be followed including proper decontamination of the work area, proper storage of reagents, ensure no contamination of reagents and cross contamination. Please refer to all necessary laboratory protocols and procedures. Collect all the required reagents and assemble them in the clean area. All the primers should be at working concentration of 10 µM.

PCR procedure with regular master mix

1. Prepare reaction master mixes in multiples of 20 µL depending on your needs, making sure you calculate how much reaction master mix is needed to complete the experiments.

2. Prepare reaction master mix as follows in a final volume of 20 µL or multiple thereof. After mixing all the components, slightly pulse vortex [10 sec] and then centrifuge to collect all the liquid to the bottom of the micro centrifuge tube.

3. Add the following reagents to the micro centrifuge tube to create the reaction master mix:

a. 11 µL Quantifast Master Mix

b. 1 µL PLU3F primer [10 µM]

c. 1 µL PLU3R primer [10 µM]

d. 7 uL of dH20

4. Prepare tubes for the qPCR run. Use 8 wells strip[s] or the plate. If you are running less than 48 reactions, it is advisable to use strips.

5. Reserve wells for 2 NTC reactions and 2 positive controls

6. While still on the clean station [hood], dispense 4 µL of the reaction master mix in each well of the tube or plate following the experiment layout which should be clearly laid out before hand

7. Add 1µL dH2O in the NTC wells while still at the clean station and seal the NTC wells using the correct transparent caps. It is important to make sure the caps snap into place and that a complete seal of the well which contains the assay is accomplished.

8. Once you are done, carry the plate from the clean station to DNA loading station.

9. Make sure your sample DNA is well prepared, clearly labeled and well organized on the work station on a rack.

10. Carefully using molecular laboratory techniques, add 1 µL sample DNA to each well.

11. After the DNA is added to each well, seal the wells using the correct caps. It is important to make sure the caps snap into place and that a complete seal of the well which contains the assay is accomplished.

12. Spin the strips or the plate at low speed for about one minute to ensure that all the reactants in each well are all the way to the bottom and to remove bubbles in the reaction

13. Load the reactions into the real-time PCR for analysis

14. The set-up the thermo cycling conditions are as follows:

a. 5 min at 96°C

b. 10 seconds at 96°C repeated for 40 cycles

c. 30 seconds at 60°C

15. Run gel to visualize amplification

PCR procedure with beads

1. Prepare reaction master mixes in multiples of 24 µL depending on your needs, making sure you calculate how much reaction master mix is needed to complete the experiments

2. Prepare reaction master mix as follows in a final volume of 24 µL or multiple thereof. After mixing all the components, slightly pulse vortex [10 sec] and then centrifuge to collect all the liquid to the bottom of the micro centrifuge tube.

3. Add the following reagents to each of the 8 well strips containing the beads to create the reaction master mix:

a. 23 µL dH20

b. 0.5 µL PLU3F primer [10 µM]

c. 0.5 µL PLU3R primer [10 µM]

4. Prepare tubes for the qPCR run. Use 8 wells strip[s] or the plate. Tubes can be labeled on the side as long as the location of the label will not interfere with reading of the well by the real-time PCR system. If you are running less than 48 reactions, it is advisable to use strips.

5. Reserve wells for 2 NTC reactions and 2 positive controls

6. Add 1µL dH2O in the NTC wells while still at the clean station and seal the NTC wells using the correct transparent caps. It is important to make sure the caps snap into place and that a complete seal of the well which contains the assay is accomplished.

7. Once you are done, carry the plate from the clean station to DNA loading station.

8. Make sure your sample DNA is well prepared, clearly labeled and well organized on the work station on a rack

9. Carefully using molecular laboratory techniques, add 1 µL sample DNA to each well.

10. After the DNA is added to each well, seal the wells using the correct transparent caps. It is important to make sure the caps snap into place and that a complete seal of the well which contains the assay is accomplished

11. Spin the strips or the plate at low speed for about one minute to ensure that all the reactants in each well are all the way to the bottom and to remove bubbles in the reaction

12. Load the reactions into the PCR machine for amplification and set up thermocycling conditions as follows:

1.12.1. 5 min at 95°C

1.12.2. 30 seconds at 95°C

1.12.3. 45 seconds at 51.9°C repeated for 40 cycles

1.12.4. 45 seconds at 72°C

1.12.5. 10 minutes at 72°C

1.12.6. Hold at 4°C

13. Run gel to visualize amplification

Standard for Multiplexed *Plasmodium* SNP Genotyping Reactions using iPLEX GOLD for MassARRAY

Purpose

The purpose of this protocol is to perform an iPLEX GOLD SNP Genotyping analysis using the Agena Bioscience MassARRAY System. The procedure describes a multiplexed SNP reaction containing 30 SNPs run against malaria DNAs samples.

Applicability

These standards applies to all designated Quality Assurance Quality Control (QAQC) personnel, MDR Laboratory personnel, Principal Investigators (PIs), USAMRD-K Laboratory Officer, the DEID director and students working within the Malaria drug research laboratories.

Responsibilities

2.3.1 It is the responsibility of the MDR laboratory supervisor to review all SOPs.

1.3.2 It is the responsibility of MDR laboratory service personnel working on all protocols to be familiar with this SOP.

1.3.3 Technical staff is responsible for the preparation, review and updating of all SOPs relative to their daily operations.

1.3.4 QAQC officers are responsible for ensuring that all SOPs are updated annually and meet the standards outlined within this SOP.

1.3.5 Training on SOPs will be conducted upon entry into any position within MDR labs and annually for all personnel to whom the SOPs apply.

1.4 All MDR laboratory personnel within USAMRD-K are required to be knowledgeable of the procedures in this SOP.

Input/Resources/Materials/Equipment

2.4.1 96-well PCR plate Centrifuge

1.4.2 96-well PCR plate standard thermocycler

1.4.3 MassARRAY Nanodispenser RS1000

1.4.4 MassARRAY Analyzer 4

1.4.5 96-well micro plates (ABGene AB700)

1.4.6 MicroAMP clear adhesive films

1.4.7 Single pipettes

1.4.8 Mutichannel pipettes

1.4.9 Filtered pipette tips (10,20,200,1000)

1.4.10 Micro tubes (1.5ml and 2.0ml)

1.4.11 HPLC grade water

1.4.12 Clean Resin

1.4.13 3-point Calibrant

1.4.14 Absolute ethyl alcohol – 200 proof

1.4.15 SpectroCHIP G96+10

1.4.16 iPLEX Gold reagent kit

1.4.17 Custom DNA primers/Primer mix

1.4.18 Custom Un-extended Primers/primer mix

1.4.19 PCR kit

1.4.20 Plate rotator

Expected Output

Effective SNP genotyping assay

Performance Indicators

Number of successful SNP genotyping assays performed

Procedures

3.7.1 Note:

3.7.1.1 Tubes and plates with reagents are lightly vortexed and centrifuged before use.

3.7.1.2 Plates are sealed with adhesive PCR sealer when not in use. The reagents (stock, dilutions and finished cocktails in plate are stored at -20ºC when not in use).

3.7.1.3 DNA samples are stored in -20ºC when not in use

3.7.1.4 DNA samples are stored in +4ºC when in use.

3.7.1.5 Before work is done in the hoods, bench areas, pipettes in the hoods are cleaned with DNase Away, followed by 70 % ethanol and illuminated with UV for 30 minutes where necessary.

PCR

3.7.2.1 Prepare 0.5µM each primer mix, containing both F and R primers for each assay in the corresponding plex.

3.7.2.1.1 Primers for the MassARRAY assay

SNP-ID	UEP_DIR	UEP_SEQ
MDR_1034	R	TAATTGAGCGCTTTGAC
CRT_356	F	TTGTATACAAGGTCCAGCA
DHFR_22	R	TTTTTTTCCCCTCATTTTTG
DHPS_437	F	TATAGGTGGAGAATCCTCTG
MDR_184	F	CCAGTTCCTTTTTAGGTTTAT
CRT_326	F	CTTCGCATTGTTTTCCTTCTTT
DHPS_613	R	TTTTGATCATTCATGCAATGGG
CRT_371	F	ATTTTATAGGGTGATGTTGTAA
MDR_86	R	TAGGATTAATATCATCACCTAAAT
MRP1_876	R	TGGAAGGATCTAAAGATGTAAATA
CRT_271	R	TTTCTTTCCTAATTAATTCTTACGTT
MRP1_191	F	TCCATTTGTTTTTTGAAGCTCTTTTG
DHPS_581	F	ACTATTTGATATTGGATTAGGATTTG
DHFR_59	R	ATTCATTCACATATGTTGTAACTGCAC
DHFR_108	R	TTGGAATGCTTTCCCAG
DHFR_16	R	GCTTTCAACCTTACAACAT
DHPS_436_	R	GGTATAACAAAAGGACCAG
CRT_356_2	F	ccGTATACAAGGTCCAGCAA
DHFR_164	R	cccTAAACAACGGAACCTCCTA
MRP1_437	R	TCAACTATATCAGAGGAAATTG
MDR_1042	R	ggaAAACCAATAGGCAAAACTAT
MRP1_1390	R	CCCAAATATTTATAAGCCATTTAA
CRT_72	R	cTTAGCAAAAATTTTATTCATTACAC
MDR_1246	R	AACTATTGAAAATAAGTTTCTAAGAT
DHPS_436	F	GATATAGGTGGAGAATCCT
CRT_76	R	GTTTAAAGTTCTTTTAGCAAAAATT

3.7.2.2 Prepare PCR cocktail in an Eppendorf tube without adding the DNA.

3.7.2.3 Aliquot 4µl of the PCR cocktail to each one of the well with the different plexes.

3.7.2.4 Add 1µl of the sample to each of the well of the 3 plexes.

3.7.2.5 Seal plate, vortex and centrifuge the plate.

3.7.2.6 Cycle the 96-well plate on a standard thermocycler with the following program;

95ºC for 2min

44 cycles

95ºC for 30 sec

56 ºC FOR 30 sec

72 ºC for 60 sec

72 ºC for 5min

10 ºC hold

3.7.2.7 Procedures: The author describes the task or step by step procedures necessary for completion of the activity. Include definition as necessary.

3.7.2.8 References.

3.7.2.9 Forms and appendices: The author lists and attaches all reference materials such as forms, checklists, or other additional information in this section. These will serve as samples only.

3.7.2.10 Document revision and version history

3.7.2.11 Document copy control

3.7.3 SAP Reaction

3.7.3.1 Prepare the SAP mixes accordingly. The values are calculated for one final 96-well plate including the 38 % overhang.

3.7.3.2 Dispense 2µl of SAP mix into each well of the sample plate, seal, vortex and centrifuge.

3.7.3.3 Incubate sample plate on the thermocycler with the following program;

37 ºC for 40 min

85 ºC for 5 min

10ºC hold

3.7.4 Extend Reaction

3.7.4.1 Prepare the extend primer pool using the excel sheet provided and fire on the MA4 to check the intensity of the High Mass primers

3.7.4.2 Prepare the iPLEX extend cocktail accordingly. The values are calculated for each one of the plexes.

3.7.4.3 Add 2µl of the iPLEX extand cocktail to each well of the plex on the 96-well plate and mix, seal, vortex and centrifuge the plate.

3.7.4.4 Cycle the plate on a thermocycler using the following program;

94 ºC for 30 sec

94 ºC for 5 sec

52 ºC for 5 sec

80 ºC for 5 sec

Go to step 3 (5 times)

Go to step 2 (40 times)

10 ºC forever

3.7.5 Conditioning

3.7.5.1 Spread out CLEAN Resin to a 96-well dimple plate and give it 10 minutes to dry out.

3.7.5.2 Add 42µl of nuclease-free water to each well of the sample plate, seal, vortex and centrifuge.

3.7.5.3 Add CLEAN Resin by gently turning the sample head over and putting it on the dimple plate. Turn the dimple plate with the sample plate and let resin fall into wells.

3.7.5.4 Rotate plate for 30 minutes.

3.7.5.5 Centrifuge the plate at 3000g for 5 minutes.

3.7 6 Dispensing

3.7.6.1 Carry out a volume check on a Nanodispenser to find optimum dispensing speed for the sample.

3.7.6.2 Dispense the reactions on a SpectroCHIP, using the RS1000 after conducting a volume check with real sample from the plate

3.7.7 MALDI-TOF MS

3.7.7.1 Run the SpectroCHIP on a MassARRAY Typer workstation with settings for iPLEX GOLD for both flex control and SpectroAcquire

3.7.8 Risk and Opportunities

3.7.8.1 Risks

	Process	**Risks**	**Risk source**	**Mitigation**
	Standard for Multiplexed *Plasmodium* SNP Genotyping Reactions Using iPLEX Gold for MassARRAY	Failed genotyping assay	Lack of training	Training on iPLEX SNP genotyping

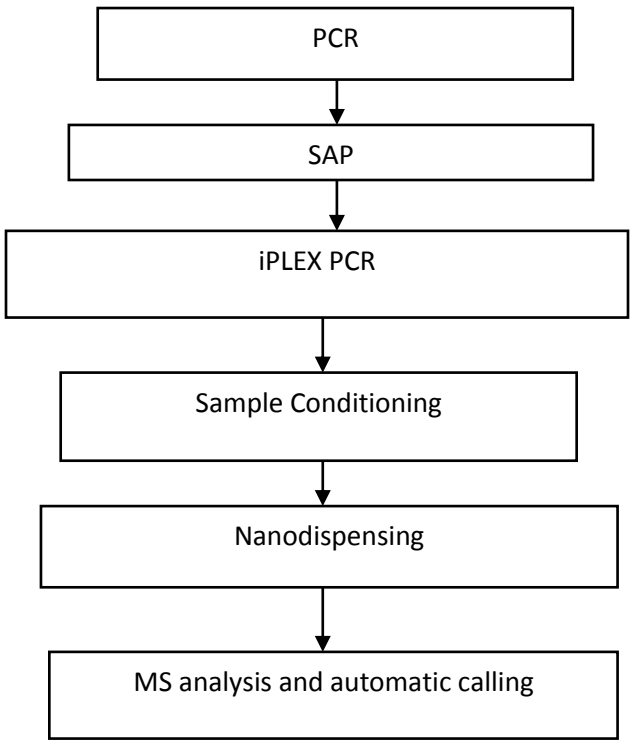
3.7.8.2 Opportunities

	Process	Opportunity	Action plan to maximize the opportunity
	Standard for Multiplexed *Plasmodium* SNP Genotyping Reactions Using iPLEX Gold for MassARRAY	Availability of guidelines iPLEX SNP genotyping	Ensure all procedures are adhered to

3.7.9 References

Regulation No, SOP etc, if applicable	Document Title
Agena Bioscience Guide	Training Instructions for Multiplexed Genotyping Reactions Using iPLEX Gold for MassARRAY

3.7.10 Flow chart



Standard operating procedures for collection, handling and DNA extraction from dried blood spots (DBS)

Purpose/Applicability

Purpose

2.4.20.1 This SOP provides instructions for Collection and DNA Extraction of Dried Blood Spots.

1.4.20.2 This SOP describes the principle rules in sample collection and handling to be able to achieve the prime goal of DNA Extraction.

Applicability

These standards apply to all designated Quality Assurance Quality Control (QA QC) personnel, Laboratory personnel, PIs and visiting students working within the MDR laboratories.

Abbreviations and Terms:

2.4.21	DNA	–	Deoxyribonucleic acid
1.4.22	DEID	–	Department of Emerging Infectious Diseases
1.4.23	MDR	–	Malaria Drug Research
1.4.24	ML	–	Milliliter
1.4.25	MM	–	Millimeter
1.4.26	PI	–	Principal Investigator
1.4.27	QA	–	Quality Assurance
1.4.28	QC	–	Quality Control
1.4.29	SOP	–	Standard operating Procedure
1.4.30	UL	–	Microlitres

Equipment and Materials:

Equipment

2.4.30.1 20 Rainin pipette Tips

1.4.30.2 100 Rainin pipette Tips

1.4.30.3 200 Rainin pipette Tips

1.4.30.4 1000 Rainin pipette Tips

1.4.30.5 Centrifuge (15^{o}-25^{o}C)

1.4.30.6 Rainin 1-10 pipette

1.4.30.7 Rainin 2-20 pipette

1.4.30.8 Rainin 50-200 pipette

1.4.30.9 Rainin 10-100 pipette

1.4.30.10 Rainin 100-1000 pipette

1.4.30.11 Lancet

1.4.30.12 Refrigerator -12 to-23^{o}c

1.4.30.13 Water Bath 56^{o}c

1.4.30.14 Water Bath 70^{o}c

1.4.30.15 Water Bath 85^{o}c

1.4.30.16 Vortex

Materials

2.4.30.17 Alcohol Pad

1.4.30.18 Absolute Ethanol

1.4.30.19 Buffer AE

1.4.30.20 Buffer AL

1.4.30.21 Buffer ATL

1.4.30.22 Buffer AW1

1.4.30.23 Buffer AW2

1.4.30.24 Gauze Pads

1.4.30.25 Proteinase K

1.4.30.26 Whatman filter papers (FTA Card Type)

1.4.30.27 Zip lock bags

Specimen

2.4.31 Whole blood

Responsibilities

2.4.32 It is the responsibility of all laboratory service personnel working on the epidemiology of malaria and drug sensitivity patterns in Kenya protocol to be familiar with and follow this SOP.

1.4.33 Technical staff is responsible for the preparation, review and updating of all SOPs relative to their daily operations.

1.4.34 QAQC officer(s) are responsible for ensuring that all SOPs are updated annually and meet the standards outlined within this SOP.

1.4.35 It is the responsibility of the MDR personnel to ensure that anyone joining the MDR protocol is well acquainted and up-to-date with this SOP.

Procedures

Sample collection through Finger Prick

2.4.35.1 Warm the Puncture site to assure good circulation.

1.4.35.2 Rub site with alcohol pad in a circular pattern to remove dirt and epithelial debris, to increase circulation, and to render the area reasonably disinfected. Let air-dry. If blood mixes with any remnant alcohol, it may cause erroneous test results.

1.4.35.3 While making the puncture apply gentle pressure while holding the skin taut. Using a sterile lancet, make the puncture perpendicular to the lines of the fingerprint. Either the left or right side of the finger may be used, depending on which results in more free-flowing wound.

1.4.35.4 Wipe away the first drop of blood that appears.

1.4.35.5 Blood must be free flowing. Squeezing the puncture site may result in specimen hemolysis and/or introduce excess tissue fluids.

1.4.35.6 Using a micropipette collect 50ul of blood

1.4.35.7 After Collection of the specimens, hold dry, sterile gauze over the wound until bleeding stops. Put band-aid over site

1.4.35.8 A few drops of blood are transferred onto a Whatman filter paper by blotting on 3 different spots and placed on the labelled zip lock bag and let to dry

1.4.35.9 The already dried filter paper is then transferred in its respective (already) labelled Zip lock bag and stored in dry place.

1.4.35.10 The Blood sample on the filter paper must be stored at room temperature, kept dry and transported to the laboratory for Molecular analysis.

DNA extraction

2.4.35.11 Place 3 punched-out circles from a dried spot into a 1.5 ml centrifuge tube and add 180 ul of Buffer ATL. Cut 3mm (1/8 inch) diameter punches from a dried blood spot with a single-hole paper punch.

1.4.35.12 Incubate at 85^{o}c for 10minutes.Briefly Centrifuge to remove drops from inside the lid.

1.4.35.13 Add 20ul Proteinase K stock Solution, mix by Vortexing, and incubate at 56^{o}c for 1h. Briefly Centrifuge to remove drops from inside the lid.

NOTE: The addition of Proteinase K is essential.

4.5.2.4 Add 200ul Buffer AL to the sample, mix thoroughly by Vortexing, and incubate at 70^{o}c for 10min. Briefly Centrifuge to remove drops from inside the lid. In order to ensure efficient lysis, it is essential that the sample and Buffer AL are mixed immediately and thoroughly.

NOTE: DO NOT ADD PROTEINASE K AFTER BUFFER AL.

A white precipitate may form when buffer AL is added to the sample. In most cases, the precipitate will dissolve during incubation. The precipitate does not interfere with the QIAamp procedure or with any subsequent application.

4.5.2.5 Add 200ul ethanol (96-100 %) to the sample, and mix thoroughly by Vortexing. Briefly Centrifuge to remove drops from inside the lid.

NOTE: It is essential that the sample and ethanol mix thoroughly.

2.4.35.6 Carefully apply the mixture from step 5 to the QIAamp spin column (in a 2ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000×g (8000rpm) for 1 min. Place the QIAamp Spin column in a clean 2ml collection tube

(provided), and discard the tube containing the filtrate. Close each spin column in order to avoid aerosol formation during centrifugation.

1.4.35.7 Carefully open the QIAamp spin column and add 500ul Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000× g (8000rpm) for 1 minute. Place the QIAamp spin column in a clean 2ml collection tube (provided), and discard the collection tube containing the filtrate.

1.4.35.8 Carefully open the QIAamp spin column and add 500ul Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000× g; 14000 rpm) for 3min.Continue directly with step 9, or to eliminate any chance of possible Buffer AW2 carryover, perform step 8a, and then continue with step 9.

NOTE: Residual Buffer AW2 in the eluate may cause problems in downstream applications. Some centrifuge rotors may vibrate upon deceleration, resulting in the flow-through, which contains Buffer AW2 coming into contact with the QIAamp spin column. Removing the QIAamp spin column and collection tube from the rotor may also cause flow-through to come into contact with the QIAamp spin column. In these cases, the optional step 8a should be performed.

(Optional): place the QIAamp spin column in a new 2ml collection tube (not provided) and discard the collection tube with the filtrate.

Centrifuge at 20,000 × g (14,000 rpm) for 1 minute.

2.4.35.9 Place the QIAamp spin column in a clean 1.5ml micro centrifuge tube (not provided) and discard the collection tube containing the filtrate. Carefully open the QIAamp spin column and add 150ul Buffer AE or distilled water. Incubate at room temperature for 1 minute, and then centrifuge at 6000× g (8000 rpm) for 1min.

NOTE: Do not elute the DNA with volumes of less than 100ul.

Three punched-out circles (3mm diameter) typically yield 150ng and 75ng of DNA from anticoagulated and untreated blood, respectively. If the yield from untreated blood is not sufficient, use 6 circles per prep instead of 3.

2.4.35.10 The Extracted DNA is then stored at-12 to-23^{o}c.

DNA Extraction alternative methods

3.5.3.1 Use the manufacturer's instructions

References

Regulation No, SOP etc, if applicable	**Document Title**
QIAamp DNA Mini and Blood Mini Handbook	DNA Purification from Dried Blood Spots (QIAamp DNA Mini Kit).

Standard Operating Procedure for Performing Conventional Polymerase Chain Reaction (PCR)

Purpose/ Applicability:

Purpose

This standard operating procedure describes how to carry out conventional polymerase chain reaction (PCR) and gel electrophoresis. PCR is a cyclic reaction that relies on sequential temperature changes which necessitate deoxyribonucleic acid (DNA) amplification. Conventional PCR is endpoint PCR which relies on gel electrophoresis for validation of resultant amplicons. This SOP gives an overview of how to perform PCR using the specified Taq polymerases:

2.4.35.11 **Amplitaq Gold DNA (Applied Biosystems) -** It is ideal for Hot start PCR which is useful when amplifying low copy number target DNA sequences.

1.4.35.12 **Amplitaq DNA polymerase with GeneAmp® 10X PCR Buffer II & MgCl2 Solution-** similar to Amplitaq Gold polymerase however it is not suitable for low copy number DNA sequences but for standard DNA amplification

1.4.35.13 **One Taq Hot Start 2x Mater Mix with Standard Buffer (New England biolabs)** This is a master mix formulation which contains Taq and deep vent polymerases, dNTPs, mgcl2 and buffer components, requiring only the addition of water, primers and DNA template for robust amplification. The 3′→5′ exonuclease

activity of Deep Vent DNA Polymerase increases the fidelity and robust amplification of *Taq* DNA polymerase

1.4.35.14 **Illustra PuReTaq PCR Beads-** These are pre-mixed, pre-dispensed, single-dose reactions optimized for performing standard PCR amplifications. They are pre-formulated to ensure greater reproducibility between reactions, minimize pipetting steps, and reduce the potential for pipetting errors and contamination. The only additional reagents required are water, primers, and template DNA. The beads are provided pre-dispensed into either 0.2 ml or 0.5 ml PCR tubes. When a bead is reconstituted to a 25 µl final volume, the concentration of each dNTP is 200 µM in 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl and 1.5 mM $MgCl_2$.

5.1.2 Applicability

The designated quality assurance/ quality control (QA/QC) personnel, laboratory personnel, laboratory supervisors, principal investigators (PI), USAMRD-K laboratory officer and the DEID director.

Abbreviations and Terms:

2.4.36 % – Percent

1.4.37 ^{0}C – Degrees Celcius

1.4.38 Dh_20 – Distilled water

1.4.39 CDC – Centre for disease control

1.4.40 DEID – Department of Emerging and Infectious Diseases

1.4.41 DNA – Deoxyribonucleic acid

1.4.42 DNTPs – Deoxynucleotide triphosphates

1.4.43 Etbr – Ethidium Bromide

1.4.44 Fwd – Forward

1.4.45 GC– Guanine and Cytosine content

1.4.46 IAW – In Accordance With

1.4.47 IATA – International Air Transport Association

1.4.48 MDR – Malaria Drug Research

1.4.49 $MgCl_2$ – Magnesium Chloride

1.4.50 min – Minute

1.4.51 NTC– Negative Template Control

1.4.52 nM – Nano Molar

1.4.53 uM – Micro Molar

1.4.54 PCR – Polymerase Chain Reaction

1.4.55 *Pf – Plasmodium falciparum*

1.4.56 PI – Principal Investigator

1.4.57 QA– Quality Assurance

1.4.58 QC – Quality Control

1.4.59 Rpm – Revolution per minute

1.4.60 SOP – Standard Operating Procedure

1.4.61 TBE – Tris Borate Ethylenediaminetetraacetic acid

1.4.62 Temps. – Temperatures

1.4.63 USAMRU-K – United States Army Medical Research Unit Kenya

1.4.64 UV– Ultra violet

EQUIPMENT AND MATERIALS:

Equipment

2.4.64.1 Applied Biosytems 7500 Fast ABI Prism system

1.4.64.2 Applied Biosytems Optical Caps (8 Caps/Strip)

1.4.64.3 ART pipette Tips (20E, 100E, 200E and 1000E)

1.4.64.4 Computer with SDS Software

1.4.64.5 Eppendorf 1-10 pipette

1.4.64.6 Eppendorf 2-20 pipette

1.4.64.7 Eppendorf 50-200 pipette

1.4.64.8 Eppendorf 10-100 pipette

1.4.64.9 Eppendorf 100-1000 pipette

1.4.64.10 Ice bucket

1.4.64.11 MicroAmp optical 96-well reaction plate.

1.4.64.12 PCR work station

1.4.64.13 Printer

1.4.64.14 Vortex

Reagents

2.4.64.15 10 X buffer A
1.4.64.16 DNTPs
1.4.64.17 Magnesium Chloride
1.4.64.18 Forward Primer
1.4.64.19 Reverse Primer
1.4.64.20 Taq Polymerase

Materials

2.4.64.21 Amber 1.5 ml microtubes, Eppendorf
1.4.64.22 ART pipet tips, sterile
1.4.64.23 Conical Tubes, 15 mL
1.4.64.24 Gloves
1.4.64.25 Micro centrifuge tubes

Samples

DNA extracted from pre-culture *Plasmodium falciparum* Parasitized blood spots or DNA extracted from whole blood samples

Responsibilities

2.4.65 It is the responsibility of all staff to follow SOPs that impact the research activities they perform.
1.4.66 Technical staff are responsible for the preparation, review and updating of all SOPs relative to their daily operations.
1.4.67 QA/QC officers are responsible for ensuring that all SOPs meet the standards outlined within this SOP and are updated annually.
1.4.68 Training on SOPs will be conducted upon entry into any position within DEID labs and annually for all personnel to whom the SOPs apply
1.4.69 All DEID laboratory personnel within USAMRD-K are required to be knowledgeable of the procedures in this SOP.

Procedures

Important notes

2.4.69.1 Ensure that all PCR reagents thaw on ice. Gently mix them at low velocity using a vortex for 30 seconds and spin down in a micro centrifuge at 8000 rpm for 1 minute before pipetting.

1.4.69.2 Lyophilized primers should be reconstituted into an initial stock concentration of 100uM of which 10uM will be aliquoted to give a primer working concentration

1.4.69.3 PCR annealing temperature is dependent upon the primers melting temperature and can be calculated using the formulae below:

81.5+0.41(%GC)-(675/N) of FORWARD primer + 81.5+0.41(%GC)-(675/N) of REVERSE primer

Where; %GC is the percentage GC on the primer sequence,

N is the total number of bases in the primer sequence.

2.4.70 Caution: Refer to GEI SOP. 396 version .01. when handling Ethidium bromide

1.4.71 All DNA should be manipulated in an area separate from master mix assembly and DNA addition, prior to addition. Assemble the master mix in the PCR work station

1.4.72 Disinfect the PCR Workstation thoroughly with RNAway or equivalent. Let the surface dry. The workstation should then be exposed to ultraviolet light for 10 minutes

1.4.72.1 Make sure to have wells for a negative control NTC reactions and 2 positive controls

1.4.72.2 All assays should be run in duplicate; samples and controls are loaded twice into two separate adjacent wells or PCR tubes

1.4.72.3 The amount of DNA template to include in a PCR mixture is dependent on DNA content and PCR assay type but optimal volume is 1ul

1.4.72.4 After PCR is complete, Gel Electrophoresis is employed so as to separate amplicons based on their size/molecular weight. It involves preparation of an agarose Gel which acts as a sieve, separating different sized fragments while the electric current from the power source provides the driving force. Percentage of Agarose in the gel is dependent of the amplicons size; larger fragments will require a lesser percentage of agarose and vice versa

1.4.72.5 With regard to gel electrophoresis, Sodium hypochlorite can NEVER be used to decontaminate the working areas where Etbr has been used for DNA visualization

1.4.73 Preparation of master mix using the Amplitaq Gold DNA and Amplitaq DNA polymerase with GeneAmp® 10X PCR Buffer II & MgCl2 Solution, PCR Master Mix kit (Applied Biosystems).

5.5.5.1 Amplitaq Gold DNA

Amplitaq Gold DNA polymerase

Reagents	Final concentration	Volume per rxn

10xPCR Gold Buffer	1×	2.5
25 mM MgCl2	1.0-4.0mM	2-8ul
10 mM dNTPs	200uM for each dNTP	4.0ul
Forward Primer	0.2-5.0uM	1-5ul
Reverse Primer	0.2-5.0uM	1-5ul
Amplitaq Gold DNA polymerase	1.25 Units/reaction	0.25
Template DNA		Variable (0.5-1ul)
PCR DH20	-	to 25ul
TOTAL		**25.0**

Cycling Conditions

	Temp (^{0}C)	**Duration**
Enzyme activation	95^{0}C	5 minutes
Initial Denaturation	95^{0}C	15 seconds
Annealing	37-72 ^{0}C	60 seconds
Initial extension	72^{0}C	60 seconds
Final extension	72^{0}C	7 minutes
Hold	4	∞

NB:

- Ideal number of cycles should be between 30-45, with 40 being the optimal number of cycles
- Any combination of water and template can be used as long as the total volume of the master mix, sample and primers equal 25 µL.

5.5.5.2 One Taq Hot Start 2x Mater Mix with Standard Buffer (New England biolabs)

One Taq Hot Start 2x Mater Mix with Standard Buffer

Reagents	**Final Concentration**	**Volume per reaction**
One Taq hot start	1×	12.5ul
Forward Primer	0.2-5.0uM	1-5ul
Reverse Primer	0.2-5.0uM	1-5ul
Template DNA	-	variable

Nuclease free DH_20		to 25ul
TOTAL		**25.0**

Cycling conditions

	Temp (^{0}C)	**Duration**
Enzyme activation	94^0C	30 seconds
Initial Denaturation	94^0C	15 -30 seconds
Annealing	37-68 ^{0}C	60 seconds
Initial extension	68^0C	60 seconds
Final extension	68^0C	5 minutes
Hold	4	∞

5.5.5.3 Illustra PuReTaq PCR Beads

Illustra PuReTaq PCR Beads		
Reagents	**Final Concentration**	**Volume per rxn**
Forward Primer	0.2-5.0uM	1-5ul
Reverse Primer	0.2-5.0uM	1-5ul
Nuclease free DH_20	-	to 25 ul
Template DNA		variable
TOTAL		**to 25.0ul**

NB:

- Ideal number of cycles should be between 35-45, with 40 being the optimal number of cycles
- PCR beads being a pre dispensed master mix formulation, one will have to add into the tube water, both forward and reverse primers, template and lastly nuclease free distilled up to a total final volume equal to 25 μL.

2.4.73.4 Prepare tubes or PCR plate for the cyclic reaction, by labeling them as per sample identity and arranging them systematically on a sample rack. If running more than 48 samples, one should use the ABI 96 well plate, which should be properly labeled reflecting sample identity

1.4.73.5 While still on the PCR workstation, dispense 24µL of the reaction master mix in each well of the tube or plate following the experiment layout which should be clearly laid out beforehand.

1.4.73.6 Add 1µL nuclease free dH_2O in the NTC wells while still at the PCR workstation and seal the NTC wells using the correct transparent caps. It is important to make sure the caps snap into place and that a complete seal of the well which contains the assay is accomplished

1.4.73.7 Once you are done, carry the plate from the PCR workstation to DNA loading area

1.4.73.8 Make sure your template is well prepared, clearly labeled and well organized on the work station on a rack

1.4.73.9 Carefully using a calibrated pipet, add 1 µL DNA to each well

1.4.73.10 After the DNA is added to each well, seal the wells using the correct transparent caps. It is important to make sure the caps snap into place and that a complete seal of the well which contains the assay is accomplished

1.4.73.11 Spin the strips or the plate at low speed (2500-3000 rpm) for about one minute to ensure that all the reactants in each well are all the way to the bottom and to remove bubbles in the reaction

1.4.73.12 Visually observe the strips or the plate by holding them high and observing at about 1-2 o'clock angle

1.4.73.13 Load the reaction tubes/ or plate into the 9700 GenAmp thermo cycler plate holder, close the lid and key in cycling conditions depending on assay type.

5.5.6 PCR Reagent optimization

Optimizing reactions for each primer-template pair may be necessary.

Achieve optimization by following the suggested guidelines for designing the primers and by varying the concentration of the following reagents:

- Template
- Primer
- MgCl2

- dNTPs
- Enzyme

The effect of these variations can be monitored by examining the intensity and distribution of amplification products after electrophoresis on agarose followed by visualization with ethidium bromide or SYBR green staining of the gel.

5.5.6.1 Optimizing template concentration

- The DNA segment to be amplified from the template can be
- 5 to 10 kb long, although 100 to 1000 bases are more typical and easier to amplify.
- If the target DNA concentration is low, more than 35 cycles may be required to produce sufficient product for analysis. As few as 1 to 10 target copies can be amplified. Validation for low copy number amplifications is best done for an average of 5 to 10 target molecules per sample to avoid statistically arising dropouts (false negative)

5.5.6.2 Optimizing the Primer Concentration

Use the following guidelines to optimize the primer concentration:

- Determine optimal primer concentrations empirically by testing concentrations in the range of 0.1 to 1.0 uM.

– Primer concentrations that are too low result in little or no PCR product.

– Primer concentrations that are too high may result in amplification of non-target sequences.

- Primer concentrations in the range of 0.2 to 0.5 uM work for most PCR amplifications.
- Reducing each primer concentration (for example, to 0.2 uM) helps reduce amplification of nonspecific products.

5.5.6.3 Optimizing the MgCl2 Concentration

The magnesium ion concentration required for optimal PCR amplification depends on the specific set of primers and template.

Too much or too little MgCl2 reduces amplification efficiency or results in amplification of non-target sequences. The optimal MgCl2 concentration must be determined empirically.

• Use the 25 mM MgCl2 supplied to adjust the magnesium ion concentration.

• Vary the concentration of MgCl2 around a midpoint of 2.5 mM. A typical range is 1.0 to 4.0 mM.

Raise the MgCl2 concentration in the reaction mix proportionately if the samples contain EDTA, citrate, or other chelators.

• Adjust the MgCl2 concentration in parallel with significant changes in the concentration (higher or lower) of sample DNA or dNTPs to keep the free magnesium ion constant. For example, reduce the concentration of dNTP from 200 uM each to 40 uM each, and reduce the MgCl2 concentration to 640 uM.

5.5.6.4 Optimizing the dNTP Concentration

The dNTP concentration provided for the Reaction Mix is balanced:

If the blend is altered and the concentration of any one dNTP is significantly different from the rest, then Taq Polymerase, will tend to misincorporate, slow down and/or terminate prematurely. Lower concentrations of dNTPs (40 uM) favor increased polymerase fidelity.

5.5.6.5 Optimizing the Enzyme Concentration

Increasing the AmpliTaq Gold DNA Polymerase, LD concentration up to 2X the recommended amount may improve the yield of amplification product.

5.5.6.6. BSAT / IAT Storage

- All blood samples suspected to harbor biological select agent should not be stored in MDR freezers or liquid nitrogen tanks. They should be sent to CDC immediately for storage and further processing. Employees should be current on the CDC select agent list. The list should be available to employees in the Laboratory. Employees should sign on training log as a proof of their knowledge on select agents.

- IAT will be kept in very limited amounts if required for quality control only. This should receive initial approval from the director and the PI

5.5.6.7 QC checks for PCR

Quality control of Primers and probes.

This is done monthly to compare the quality of results generated by different batches of primers and probes, and to monitor efficacy of diluted primers and probes over time.

This comprises setting up a PCR reaction with both positive and negative controls as templates. *Plasmodium falciparum* clones; 3D7, W2, D6, DD2, HB3, V1S serve as positive control, which represents either wild or mutant type of a molecular marker. Yielding representative amplicons (either wild or mutant type) per clone is a sure indicator of optimum primer (s) capability.

Lastly comparing results generated for the different batches of primers and probes. Comparable results certify their efficiency however in the event of a discrepancy; the procedure is repeated three times with the same materials and conditions but with different technicians. If Comparable results are obtained, then the first discrepancy will be attributed to the Technician and not the primers and probes. If Discrepancies still develop after the three test runs, then the set of Primer(s) that is (are) unable to reproduce the expected range of results for the two clones, is (are) considered redundant.

Quality Control of the PCR Assay.

Clean Laboratory Conditions

This is essential as PCR is capable of detecting and amplifying a single molecule of DNA, so as to prevent Contaminations.

This is achieved by always wearing fresh gloves, use sterile glassware, tubes and pipette tips and sterile solutions and the work area is properly sterilized with Ethanol, 70 % before, during and after the procedure.

Use of both Positive and Negative controls as templates.

These controls are a means of verifying Quality of the PCR Reaction.

The above mentioned *Plasmodium falciparum* clones are run together with DNA from field isolates and are expected to give a predicted result per molecular marker and assay type, hence achieving this range certifies the Quality of the Assay.

Negative control comprises of distilled water as template, this is to ensure that the results of the assay are truly due to amplification of the right samples.

Quality Control of Personnel/Technician.

Training of Personnel on PCR Assay

Technicians carrying out PCR Assays are trained on carrying out the assays, by first going through this SOP and are also checked for reproducibility of results.

Internal Checking System: The laboratory supervisor is responsible of ensuring that it has been implemented at least quarterly.

This is done by assigning the PCR assays to various technicians and comparing results generated by each technician. Technicians whose Assays do not give the expected results are further trained and monitored for reproducibility of results.

References

Regulation No, SOP etc, if applicable	**Document Title**
Birch, D. E., Kolomodin, L., Laird, W. J (1996).	Simplified Hot Start PCR. Product review in *Nature* 381: 445-446.
Chou, Q., Russell, M., Birch, D.E., Raymond, J., and Bloch, W. (1992).	Prevention of pre-PCR mis-priming and primer dimerization improves low- copy-number amplifications. *Nucleic Acids Res*. 20:1717–1723.