TEMPORAL AND SPATIAL TRENDS OF *PLASMODIUM FALCIPARUM* MULTI-DRUG RESISTANCE PROTEIN 1 GENE MUTATIONS DURING IMPLEMENTATION OF ARTEMISININ COMBINATION THERAPIES BETWEEN 2008 AND 2019 IN KENYA

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A RESEARCH THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF A DEGREE OF MASTER OF SCIENCE IN MEDICAL BIOTECHNOLOGY

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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.

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DEDICATION

I dedicate this work to the Heavenly Father for being my all-time comforter, encourager and the source of my strength, also to my family for the motivation and offering overwhelming support throughout the entire period.

ACKNOWLEDGEMENTS

I acknowledge Almighty God for his everlasting protection, direction and guidance. Also for granting me the wisdom, dedication, patience, strength and determination to carry out this work in both challenging and good times. I would like to thank my supervisors, Prof. Collins Ouma, Dr. Hoseah Akala and Dr. Ben Andagalu for their technical and moral support. I thank my family for being my support system. All the members of the Malaria Drug Resistance laboratory at the United States Army Medical Research Directorate Africa-Kenya for their overwhelming support for the entire period of my research and assisting me in all the stages of the work, especially Major Amanda Roth, Dr. Ben Andagalu, Dr. Hoseah Akala, Luicer Ann, Dennis Juma and Benjamin Opot for providing deep insights onto the study, Raphael Okoth in training to run the assays, Redemptah Yeda in spearheading analyses, my friends Oliver Towett for assisting in graphing and David Abuom for linking me to be part of the team. I am immensely grateful for all their contributions and help and will forever be indebted to them.

ABSTRACT

Single nucleotide polymorphisms (SNPs) in the *Plasmodium falciparum* multi-drug resistance protein 1 (*Pfmrp1*) gene have previously been associated with conferring resistance against artemisinin and its partner drugs in Southeast Asia (SEA) and there are concerns of resistance spreading to Africa as the previous patterns. With no suitable replacement for artemisinin combination therapies (ACTs), establishing the frequency of these polymorphisms contributing to impaired response to ACTs is key for continued drug resistance surveillance in Africa, where the few putative *Kelch 13* propeller polymorphisms reported in Rwanda and the horn of Africa have not affected ACTs tested and used there. Apart from *Kelch 13, Pfmrp1* gene is also a potential drug resistance marker neglected in Kenya as compared to chloroquine (CQ) and sulfadoxine pyrimethamine (SP) targets. The frequency of *Pfmrp1* SNPs associated with anti-malarial resistance and correlation with *in vitro* drug sensitivities from Kenyan parasite isolates between 2008 and 2019 is not known. Of particular interest between 2008 and 2019 was a representation of the transition period and post-ACTs timelines of the study period, to compare the trends over time to help understand the prevalence of resistance. Therefore the general objective of this study was to investigate the temporal and spatial trends of the *Pfmrp1* gene mutations during the implementation of ACTs between 2008 and 2019 in Kenya. The specific objectives were to determine the frequency of SNPs of *Pfmrp1* gene, determine *in vitro Plasmodium falciparum (P.f.)* drug response patterns and establish the correlation between the polymorphic versus wild-type and *in vitro* anti-malarial response profiles for the Kenyan field isolates collected between 2008 and 2019. In a cross-sectional retrospective study of 6 months and older participants, 300 samples collected from 6 sites across Kenya namely; Kisumu, Kombewa, Malindi, Marigat, Kisii and Kericho between 2008 and 2019 under an ongoing, epidemiology of malaria and drug resistance patterns in Kenya study, were assayed for SNPs in *Pfmrp1* gene codons; H191Y, S437A, I876V and F1390I using Agena MassARRAY platform. Field isolates were also tested against standard antimalarials selected; artemisinin (ART), lumefantrine (LU), amodiaquine (AQ), mefloquine (MQ), quinine (QN) and CQ to determine their *in vitro* drug sensitivity using the malaria SYBR Green I-based fluorescence assay. Categorical data was analyzed as proportions, while the continous data was reported as median IC_{50} values. Of the 300 samples typed, polymorphisms at *Pfmrp1* codon I876V was the most frequent at 58.9% (162/275) mutants followed by F1390I, 7.1% (19/267) and S437A, 3.3% (9/274) while H191Y was the least at 3.1% (5/151). The antimalarial sensitivity patterns of AQ and QN were shown to have median IC_{50}s that increased over time between 2008 and 2019 from 2.959ng/ml [IQR=2.453-4.189, n=47] and 1.967ng/ml [IQR=1.332-3.243, n=54] to 7.111ng/ml [IQR=6.562-9.054, n=11] and 3.046ng/ml [IQR=2.178-6.175, n=20] respectively. MQ appeared to be undergoing positive selection, it was shown to have median IC_{50}s that increased resistance over time from 16.59ng/ml [IQR=12.13-35.96, n=16] to 20.32ng/ml [IQR=12.54-23.12, n=5] and it increased significantly (P<0.0001) during the same period. However, LU showed contradicting results of increased sensitivity and resistance over time during the study period. The correlation of infections with mutation at codon I876V were associated with higher QN and LU with 50% inhibition concentration during *in vitro* tests, suggesting reduced sensitivity. Identifying these markers are critical to understanding and tracking other rising prevalence of ACTs resistance within this region after its implementation besides, is essential as prerequisites for any control and elimination programs. Study findings showed early indicators signaling resistance patterns to ACTs and selection should be tracked. *Pfmrp1* gene mutations should also be tracked as an important candidate gene for monitoring drug resistance in Kenya.

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ABBREVIATIONS AND ACRONYMS

ACT/ACTs	Artemisinin Combination Therapy/Therapies
AL	Artemether-lumefantrine
AM	Artemether
ART/ARTs	Artemisinin/Artemisinin Derivatives
AS-AQ	Artesunate-Amodiaquine
AS-MQ	Artesunate-Mefloquine
AS-SP	Artesunate- Sulfadoxine pyrimethamine
CQ	Chloroquine
dH_2O	Distilled water
DHA-PQ	Dihydroartemisinin-Piperaquine
dNTPs	Deoxyribonucleotide triphosphates
GMS	Greater Mekong Sub-region
IC_{50s}	50% Inhibitory concentration
IPLEX	Increased Plexing Efficiency and Flexibility
KCH	Kericho
KDH	Kisumu
KOM	Kombewa
KSI	Kisii
LU	Lumefantrine
MALDI-TOF MS	Matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MDH	Malindi

MSF	Malaria SYBR Green I-based fluorescence
$MgCl_2$	Magnesium Chloride
MGT	Marigat
MQ	Mefloquine
µM	Micromolar
ML	Millilitres
NaCl	Sodium Chloride
P. f.	*Plasmodium falciparum*
PCR	Polymerase Chain Reaction
Pfcrt	*Plasmodium falciparum* chloroquine Resistance Transporter
Pfdhfr	*Plasmodium falciparum* dihydrofolate reductase gene
Pfdhps	*Plasmodium falciparum* dihydropteroate synthase gene
Pfmdr1	*Plasmodium falciparum* multidrug resistance gene 1
Pfmrp1	*Plasmodium falciparum* multi-drug resistance protein 1
QN	Quinine
RBCs	Red Blood Cells
RDTs	Rapid Diagnostic Tests
RPM	Rotations per minute
SEA	South East Asia
SNPs	Single Nucleotide Polmorphisms
SP	Sulfadoxine-pyrimethamine
sSA	Sub-Saharan Africa
WHO	World Health Organisation

DEFINITION OF TERMS

Anti-malarial resistance and **treatment failure** is defined as lack of malarial parasitemia clearance or prevention of re-infection after an antimalarial drug is given even if clinical signs have subsided. In addition, the persistence of a parasite strain and multiplication (WHO, 2018).

Drug pressure- intense exposure of a population to a given drug, usually an antimicrobial agent such as an antimalarial.

Hematocrit- the ratio of the volume of packed red blood cells to the volume of whole blood as determined by a centrifugation instrument: a measure of possible anemia.

Premunition- imperfect immunity.

Selection- a process in which environmental or genetic influences determine which types of organism thrive better than others, regarded as a factor in evolution.

Selection pressures - external agents which affect an organism's ability to survive in a given environment, can be negative (decreases the occurrence of a trait) or positive (increases the proportion of a trait).

Single nucleotide polymorphism- 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.

Mutant, mixed and wild-type alleles –mutants are pure polymorphic alleles (different from the standard alleles), wild-type are the standard alleles which function normally, mixed are heterogenous (comprising of the wild type together with the mutant genotypes existing in a single sample).

CHAPTER ONE: INTRODUCTION

1.1 Background Information

Globally, there were an estimated 229 million malaria cases in 2019 in 87 malaria endemic countries, declining from 238 million in 2000, at the Global technical strategy (GTS) for malaria 2016-2030 baseline of 2015, there were 218 million estimated malaria cases (WHO, 2020). Malaria case incidence (cases per 1000 population at risk) reduced from 80 in 2000 to 58 in 2015 and 57 in 2019 globally, between 2000 and 2015, global malaria case incidence declined by 27% and between 2015 and 2019 it declined by less than 2%, indicating a slowing of the rate of decline since 2015 (WHO, 2020). This positive progress has rekindled hope that malaria elimination is a reality. From a baseline of 2015, the GTS aimed to achieve, a reduction of 40% of malaria morbidity incidence and mortality rate by 2020 but that was not achieved, the strategy aim was also to eliminate malaria in at least 10 countries and prevention of reintroduction in all countries that achieved elimination (GTS, 2015), while the gains to date are impressive the global malaria challenge remains enormous and the rate of progress is slowing, *Plasmodium* resistance to antimalarial medicines is one of the key recurring challenges in the fight against reducing malaria burden (WHO, 2019b).

The World Health Organization (WHO) African Region, with an estimated 215 million cases in 2019, accounted for about 94% of cases (WHO, 2020). In Kenya, there are an estimated 3.5 million new clinical cases and 10,700 deaths each year and those living in western Kenya have an especially high risk of malaria (CDC, 2018a). *Plasmodium falciparum* (*P.f.)* is the species most frequently associated with severe malaria and accounts for 80-90% of cases in Kenya (MOH, 1994)**.** Accomplishing the WHO milestone of eliminating malaria by 2020 has been hindered by several challenges facing malaria elimination efforts which impede control efforts (WHO, 2018).

The first cases of resistance to artemisinin and its partner drugs were reported in the Greater Mekong Sub-region (GMS) which comprises; Cambodia, the People's Republic of China (specifically Yunnan Province and Guangxi Zhuang Autonomous Region), Lao People's Democratic Republic, Myanmar, Thailand and Viet Nam followed by artemisinin combination therapies (ACTs) failure (Menard & Dondorp, 2017a). This report ushered the need for expanding and strengthening resistance surveillance systems beyond the region to monitor the emergence or dispersion of artemisinin resistance (Nsanzabana, 2019). Resistance to artemisinins has been linked to several point mutations in a propeller domain of a *Kelch 13* gene (Mbengue *et al*., 2015) although, the particular mechanism of resistance is not yet completely understood (Nsanzabana, Djalle, Guérin, Ménard, & González, 2018). There is a recent case in Rwanda that reported *Kelch 13* mutations in their populations to be associated with slow parasite clearance however, it has not affected the efficacy of ACTs tested and used there (Uwimana *et al*., 2020). There are also few countries in the horn of Africa where *Kelch 13* mutations have been reported but ACTs efficacy is also still intact in their populations (WHO, 2020). The efficacy of ACTs in Africa is therefore still high and promising as compared to GMS irregardless of *Kelch 13* mutations in some populations however, they still need close monitoring since this is where the burden lies.

Resistance to drugs has been shown to emerge in distinct regions with distinct epidemiological zones (Takala-Harrison *et al*., 2015). For chloroquine (CQ) and sulfadoxine-pyrimethamine (SP) resistance, parasite adaptive traits at gene level were parallel across all geographic locations despite discernible evidence of independent origins across transmission region based on population structure analyses (Alam *et al*., 2011). A worldwide surveillance study clarified that *Kelch 13* mutations that cause resistance in SEA exist at a lower percentage in Africa. African *Kelch 13* mutations have originated locally thus suggesting that *Kelch 13* is presently not

undergoing strong selection in Africa (Amaratunga *et al*., 2016). Studies such as that of Amaratunga and co-workers mentioned above underscore the need for public health surveys on ACT resistance to focus on other targets besides the *Kelch 13.* This will expand the strategies that hinder resistance and account for local evolutionary conditions that are evidenced to differ greatly from one geographic region to another.

The *Pfmrp1* is a member of ATP-binding cassette (ABC) transporter superfamily and an 1822–amino acid protein situated in the plasma membrane of the parasite (Dahlstrom *et al*., 2009). It imparts to parasite reactions to several anti-malarial drugs through drug efflux and plays a part in the efflux of glutathione, CQ, mefloquine (MQ) and quinine (QN) globally (Koenderink *et al*., 2009). Its association with susceptibility to antimalarial drugs and positive parasite selection makes *Pfmrp1* an amenable target for tracking resistance (Gupta *et al*., 2014a). Evidence of single nucleotide polymorphisms (SNPs) in this gene have been shown to be associated with resistance to artemisinin (ART), lumefantrine (LU), amodiaquine (AQ) and MQ (Dahlström, 2009). Polymorphism in this gene has been associated with reduced intracellular drug build-up, due to the changes in the parasite membrane protein of the gene (Veiga *et al*., 2011). Apart from *Kelch 13*, the *Pfmrp1* gene has been shown to be a potential candidate marker causing artemisinin combination therapy (ACT) resistance. In order to assess its status especially in Africa where mutations in *Kelch 13* have not shown much significance. This current study chose to establish the frequency of *Pfmrp1* SNPs in Kenya since they have not been documented and it is really essential to facilitate surveillance.

Surveillance of anti-malarial drug resistance depends on three distinct and complementary methods: *in vivo* efficacy studies to determine treatment failures to drugs, *in vitro* assessment of parasite sensitivity to drugs and evaluation of molecular markers linked to drug resistance (González, Djibrine, Philippe, Didier, & Iveth, 2018). *In vitro* drug sensitivity tests and the

parasite clearance rates are the principal measurements in determining the phenotypic response of strains to drugs though the latter is less robust and can only screen a limited number of samples due to cost (Bayih *et al*., 2017). Malaria SYBR Green I-based fluorescence (MSF) *in vitro* assay provides a consistent, cost-effective and fast alternative to previous radioisotopic approaches in sensitivity testing (Smilkstein, Sriwilaijaroen, Kelly, Wilairat, & Riscoe, 2004).

Molecular marker analyses offer a less costly scalable practical alternative for typing a large number of parasites targets (Apinjoh, Ouattara, Titanji, Djimde, & Amambua, 2019). When paired with *in vitro* data, these two methods offer critical details on drug response and could be predictive of changing patterns of drug resistance in the parasite population (Singh, Kumar, & Gupta, 2017)**.** Parasite drug resistance for several routine antimalarials have been compared to particular genetic mutations and tracking molecular markers linked with resistance provides an easy and robust tool to detect the emergence and distribution of resistant parasites (Nsanzabana *et al*., 2018). Agena MassARRAY system using polymerase chain reaction (PCR)-based single base extension has been previously described to be suitable for assaying SNPs associated with drug resistance in several molecular markers of *Plasmodium falciparum* (*P.f.*) anti-malarial resistance in Kenya (Yeda *et al*., 2016). The genotyping platform is able to multiplex up to 40 SNPs per reaction, it uses a matrix-assisted laser desorption ionization time-of-flight mass **s**pectrometry (MALDI-TOF MS) coupled with single-base extension PCR for high-throughput multiplex SNP detection and is considered cheap (Syrmis *et al*., 2011)**.** Therefore this study used the Agena MassARRAY system and the MSF *in vitro* assay to correlate the polymophisms in *Pfmrp1* gene and *in vitro* anti-malarial response profiles for the Kenyan field isolates collected between 2008 and 2019 in order to determine the trends observed during the study period. Of particular interest between 2008 and 2019 was a representation of the transition period and post-

ACTs timelines of the study period, to compare the trends over time to help understand the prevalence of ACTs resistance.

In the current study, the drugs selected as follows; ART, LU, AQ, MQ, QN, CQ and the codons; 191, 437, 876, 1390 were chosen simultaneously, it was based on the behaviourial pattern of parasites from South East Asia (SEA), Oceania and Africa at large. The mutations at the 4 codons are already established and validated non-synonymous SNPs frequent in the populations and are associated with resistance to the chosen drugs. Evidence of positive selection on the *Pfmrp1* gene and its association with reduced sensitivities of parasites to the selected antimalarials was based on the following studies (Dahlström *et al*., 2009; Gupta *et al*., 2014b; Pirahmadi, Zakeri, Afsharpad, & Djadid, 2013; Veiga *et al*., 2011; Zhao *et al*., 2019) therefore, the likelihood of the same mutations being frequent in Kenyan isolates was postulated to also be associated with the reported antimalarials as the previous studies.

1.2 Statement of the problem

Studies have shown that emerging and existing *P.f.* drug resistance prevalence have not been fully elucidated to date in all populations. The frequency of recently described SNPs in *Pfmrp1* and their role in anti-malarial drug responses have not been extensively studied in sSA as opposed to CQ and SP resistance targets. Studies have majorly concentrated on only five molecular markers named; *Plasmodium falciparum* chloroquine Resistance Transporter gene (*Pfcrt*), *Plasmodium falciparum* dihydrofolate reductase gene *(Pfdhfr*), *Plasmodium falciparum* dihydropteroate synthase gene (*Pfdhps*), *Plasmodium falciparum* multidrug resistance gene 1 (*Pfmdr1*) and *Plasmodium falciparum Kelch 13* (*Pfk13*). *Pfmrp1* being a neglected marker especially in Kenya where it has not been documented. The frequency of *Pfmrp1* SNPs associated with anti-malarial resistance and correlation with *in vitro* drug responses from Kenyan parasites between 2008 and 2019 is not known. This is an essential prerequisite in any control and elimination program set out to achieve the GTS for malaria 2016–2030 baseline of 2015, especially in endemic countries such as Kenya. Therefore, this study chose to investigate the temporal and spatial trends of *Pfmrp1* mutations during the implementation of ACTs between 2008 and 2019 as a representation of the transition period and post-ACTs timelines of the study period to compare the trends over time to help understand the prevalence of ACTs resistance in Kenya.

1.3 General objective

To determine the temporal and spatial trends of the *Plasmodium falciparum* multidrug resistance protein 1 gene mutations during the implementation of artemisinin combination therapies between 2008 and 2019 in Kenya.

1.3.1 Specific objectives

i. To determine the frequency of single nucleotide polymorphisms of *Plasmodium falciparum* multidrug resistance protein 1 gene (*Pfmrp1)* in Kenyan field isolates collected between 2008 and 2019.

ii. To determine *in vitro Plasmodium falciparum* drug response patterns of artemisinin, lumefantrine, amodiaquine, mefloquine, quinine and chloroquine in Kenyan field isolates collected between 2008 and 2019.

iii. To establish the correlation between the polymorphic versus the wild-type in the *Plasmodium falciparum* multidrug resistance protein 1 gene and *in vitro* anti-malarial response profiles for the Kenyan field isolates collected between 2008 and 2019.

1.3.2 Research questions

i. What are the frequencies of single nucleotide polymorphisms of the *Plasmodium falciparum* multidrug resistance protein 1 gene in Kenyan field isolates collected between 2008 and 2019?

ii. What are the *in vitro Plasmodium falciparum* drug response patterns of artemisinin, lumefantrine, amodiaquine, mefloquine, quinine and chloroquine in Kenyan field isolates collected between 2008 and 2019?

iii. What is the correlation between the polymorphic versus the wild-type in *Plasmodium falciparum* multidrug resistance protein 1 gene and *in vitro* anti-malarial response profiles for the Kenyan field isolates collected between 2008 and 2019?

1.4 Significance of the study

The study adds knowledge on the frequency of the *Pfmrp1* gene SNPs and their correlation with *in vitro* drug response patterns from Kenyan field isolates between 2008 and 2019 where they were unknown. The data and information generated is high quality surveillance data which benefits Kenya's National Malaria Control Program for decision making in order to drive tailored responses consistent with national or subnational goals additionally to, intensify malaria control in order to reduce the burden of the disease and delay or prevent spread of resistance in Kenya. Identifying these molecular markers is critical to help identify and track the prevalence of molecular mutations associated with ACTs resistance in Kenya after their implementation. This data benefits WHO to help guide the ministry of health in streamlining elimination efforts towards *falciparum* malaria in Kenya. Due to the absence of a vaccine in all age groups, ensuring that the recommended ACTs are effective, timely changes to national treatment policies can be implemented and resistance can be detected early, WHO needs to advise the national malaria programs to prioritize *Pfmrp1* gene as well by when tracking ACTs resistance in Kenya. Since, it has been shown to be a potential candidate marker causing ACTs resistance in this region.

CHAPTER TWO: LITERATURE REVIEW

2.1 Malaria Treatment Regime Globally

Globally, the recommended ACTs for treatment of malaria have artemisinin or its derivative combined with one or more drugs and includes; artemether-lumefantrine (AL), artesunate-amodiaquine (AS–AQ), artesunate-mefloquine (AS–MQ), artesunate-sulfadoxine-pyrimethamine (AS–SP) and dihydroartemisinin-piperaquine (DHA–PQ) (WHO, 2020). According to the WHO treatment regime in the WHO regions; African region is AL, AS-AQ, and DHA-PQ, Americas is AL, AS-MQ, and CQ, SEA is AL, AS-SP and DHA-PQ, Eastern Meditterenian is AL and AS-SP and Western Pacific is AL (WHO, 2020). Since 2016 most African countries especially East Africa use AL and AS–AQ, with some adding DHA–PQ for uncomplicated malaria, AS, AM and QN for severe malaria as a first-line treatment regime (Holmgren *et al*., 2007; Sisowath *et al*., 2009).

2.2 History of Antimalarial Drug Resistance and Drug resistance status in Africa

According to the centre for disease control and prevention (CDC), expanding malaria morbidity and mortality rates is caused by the emergence of anti-malarial drug resistance, which has become a major constraint to malaria control, resistance has now been verified in only *P.f.* and *Plasmodium vivax (P.v.)* (CDC, 2018b). CQ was the first-line treatment of malaria from the 1950s until the 1990s when resistance emerged and spread to various parts of the world, SP later replaced it however, resistance emerged swiftly and is now present at a high prevalence in most endemic zones (Plowe, 2009). A previous study has shown that resistance emerged from SEA and was widely spread to other regions including Africa eventually, ACTs became an alternative drug of treatment by the end of 2006 and is currently the first-line treatment of malaria (Kavishe *et al*., 2014). The *P.f.* resistance to ART has so far been discovered in five countries in the GMS; Cambodia, Lao People's Democratic Republic, Myanmar, Thailand and Vietnam (Sharma *et al*.,

2018). Up to date, no preventive vaccine against malaria has been developed to be effective in all age groups and malaria control relies mostly on anti-malarial drugs. Anti-malarial drug resistance has however become a major threat facing chemotherapy, it is therefore important to track any emerging resistance especially in sSA, to avoid the previous incident which led to high mortality rates in this region since it is where the brunt of the disease lies. This study therefore facilitated tracking any emerging resistance to ACTs in Kenya which also forms sSA.

According to WHO (WHO, 2019a) in various regions of Africa, a few foreign cases of therapeutic failures were described in some European migrants that were cured with DHA-PPQ or AL. Further research neither confirmed resistance nor the infections comprising *PfKelch 13* mutations hence rendering Africa resistance-free to ACTs however, a recent study in Rwanda identified these mutations in their population (Uwimana *et al.*, 2020). Reporting in these cases was crucial since resistance of therapeutic failures in migrants might be an early indicator of drug resistance (WHO, 2019a). Important tools for conserving ACT efficacy are to keep track of selection and answer to arising indicators of drug resistance (Venkatesan *et al*., 2014). The present study was set up to determine the temporal trends of the *Pfmrp1* gene mutations during the implementation of ACTs between 2008 and 2019 in Kenya in order to help track selection.

2.3 Surveillance of Antimalarial Drug Resistance

The surveillance of malaria drug resistance entails directly estimating parasite drug responses or indirectly estimating the frequency of resistance linked mutations in a parasite population (Mboup *et al*., 2013). There are four distinct techniques that assess anti-malarial drug efficacy which include; *in vivo*, *ex vivo*, *in vitro* evaluations of drug susceptibility and molecular markers that play a role in antimalarial drug resistance (Maji, 2018; Menard & Dondorp, 2017b). The emergence of drug resistance can be identified in time by *in vitro* susceptibility testing, also

changing temporal and spatial patterns of parasite drug sensitivity or changes in responses of individual drugs currently used as well (Menard & Dondorp, 2017b). Tracking the frequency of mutations associated with antimalarial resistance over a long period of time is able to unveil patterns in allele selection in a population with time and can expand the remedial life of the present and upcoming cures (Mboup *et al*., 2013). It is on that account that this study tracked resistance by estimating the frequency of resistance linked mutations in *Pfmrp1* and *in vitro* susceptibility testing in Kenyan isolates to better surveillance of ACT resistance in this region over time.

2.3.1 *In vitro* drug testing and significance of using the Malaria SYBR Green I-based fluorescence (MSF) assay approach

Sensitivity testing approaches of antimalarial agents are mainly established on *in vitro* culture of the parasite to monitor drug activity. These techniques are straight forward, easy and enable early identification of drug-resistant parasites (Maji, 2018). The assay must present a first demonstration of the pharmacological effect that is always manifested as the concentration needed to hinder the parasite growth by 50% (IC_{50}) (Vial *et al*., 2010). Furthermore, they enable every constituent of a compound to be examined separately which is essential in determining which particular drug therapy is failing in *in vivo* cases when therapeutic failures are observed (Kyle *et al*., 2007). The test is not influenced by individual confounding elements such as re-infections, weak drug assimilation, immunity, associated infections and compliance that influence *in vivo* studies. Therefore, studies have rendered it a fast cost-effective method proving it as the best option for evaluating anti-malarial drug efficacy especially in endemic zones (Kronmann *et al*., 2013). The potency of antimalarials on the viability of the parasite was measured by *in vitro* assays. Trends in susceptibility to a specific drug and an alert to the early emergence of resistance in Kenyan parasite populations was exhibited in a confined human

population by this study. In addition, the current study offered the opportunity to compare *in vitro* parasite responses from different Kenyan sites during the transition period and post-ACTs timelines of the study period and correlated with potential polymorphisms of resistance.

Malaria SYBR Green I-based fluorescence (MSF) assay is a quick, consistent and cost-effective choice to radioisotopic techniques in the assessment of drug resistance levels (Bacon *et al*., 2007; Picot *et al*., 2015). This assay determines parasite growth using a fluorescent-based method that uses SYBR Green I, it is based on the principle of using lysis buffer containing SYBR green I prior to the determination of the fluorescent values (Bacon *et al*., 2007). The SYBR Green is a skewed cyanine dye that intercalates in the genomic DNA of the malaria parasite when it intercalates into DNA, it is extremely fluorescent, absorbing light at a wavelength between 390 and 505 nm, with a peak at 497 nm and a secondary peak near 254 nm. It emits light at 505–615 nm, with a peak at 520 nm (Vossen, Pferschy, Chiba, & Noedl, 2010). Several Kenyan studies have validated the MSF assay as an ideal dependable single plate high-throughput labor and time-saving *in vitro* sensitivity test method without the costs and dangers of the other tests used in anti-malarial drug screening (Johnson *et al*., 2007; Rason, Randriantsoa, Andrianantenaina, Ratsimbasoa, & Menard, 2008; Smilkstein *et al*., 2004). Therefore it was the most suitable method in testing the sensitivities of the antimalarials in this study.

2.3.2 Significance of genotyping using the Agena MassARRAY system

MassARRAY system comprises an initial locus-specific PCR reaction, then the single base extension using mass modified dideoxynucleotide terminators of an oligonucleotide primer that binds upstream of the polymorphic site of interest instantly (Lee, Rimesso, Reynolds, Cai, & Baker, 2016). Using Matrix-assisted laser desorption ionization time-of-flight (MALDI TOF) mass spectrometry, the different mass of the extended primer detects the SNP allele (Gabriel, Ziaugra, & Tabbaa, 2009). This technique has been described as ideal for high-throughput typing

analysis in population genomic studies (Lanzaro, Stephanie, Christen, Douglas, & Gregory, 2012). The platform is multifaceted and can run multiplexed reactions having more than 40 distinct loci in a single reaction with a shorter turn-around time in data generation and analysis (Oeth, Mistro, Marnellos, Shi, & Boom, 2009). Several studies have used it in screening polymorphisms associated with anti-malarial resistance in different molecular markers (Apollo *et al*., 2018). This is a reliable tool in evaluating *Pfmrp1* SNPs associated with anti-malarial resistance in Kenya since it is proven to be a quicker and cheaper technique compared to sequencing, with high sensitivity and specificity it avoids the turn-around time involved in sequencing and bioinformatics analysis furthermore, eliminates the incompetency involved with bioinformatics analysis.

2.4 The Molecular Markers of Anti-malarial Resistance

One of the various techniques for the surveillance of anti-malarial efficacy and resistance frequency is by the use of molecular markers detection for parasite's drug resistance (Xu *et al*., 2018). *P.f.* parasites remain under ongoing selective pressure from several anti-malarial drugs and between *P.f.* genes associated with anti-malarial resistance, so far most studies have majorly concentrated on SNPs present in only five *P.f.* genes; *Pfcrt*, *Pfmdr*1, *Pfdhfr*, *Pfdhps* and *Pfk13* (Nag *et al*., 2017). Studies have shown SNPs at codons 72, 74, 75 and 76 of *Pfcrt* and 86, 184, 1034, 1042 and 1246 of *Pfmdr*1 were evidenced to be linked to parasite's chloroquine resistance in Ethiopians and Venezuelans (Golassa, Enweji, Erko, Aseffa, & Swedberg, 2014; Griffing *et al*., 2010). Furthermore, SNPs in the *Pfdhps* gene codons S436A/F, A437G, K540E, A581G and A613S/T of *P.f.* present resistance to sulfadoxine and dihydrofolate reductase *Pfdhfr* C50R, N51I, C59R, S108N and I164L to pyrimethamine in Pakistan populations (Yaqoob *et al*., 2018). *Pfk13* SNPs in codons K189T, K189N, 443, 459, 469, 473, 533, 539, 553, 556, 578, 692 have been associated with artemisinin resistance in Senegal (Talundzic *et al*., 2017) and mutations in

codons F446I, N458Y, M476I, Y493H, R539T, I543T, P553L, R561H and C580Y have been verified to be markers of resistance in SEA (Muro *et al*., 2019). These are the most studied molecular markers of resistance in sSA, however, this study explored another marker known as the *Pfmrp1* gene that enhanced understanding the prevalence of ACT resistance in Kenya.

2.5 The Trends of *Plasmodium falciparum* multidrug resistance protein 1 (*Pfmrp1)* polymorphisms and correlation with *in vitro* antimalarial drug sensitivities in the Greater Mekong subregion

Reduced sensitivity to artemisinin and its derivatives (ARTs) have been described to be linked to polymorphisms in the *Pfmrp1* gene, however the mechanism of action and resistance of ARTs is not well comprehended (Bustamante *et al*., 2012). Previous studies in various regions have shown the potential significance of *Pfmrp1* in anti-malarial drug resistance (Dahlstrom *et al*., 2009; Gupta *et al*., 2014a; Koenderink *et al*., 2009). For instance, in Iran 4 years after the introduction of ACTs in *Pfmrp1* gene, 191Y (76.5%), 437A (69.5%), 876V (64.5%) and 1390I (17%) polymorphisms associated with artemisinin resistance were found in their populations (Pirahmadi *et al*., 2013). A study conducted in the China-Myanmar border associated SNPs at codons; N325S, H785N, T1007M, F1390I, I876V as well as H191Y and S437A with reduced *in vitro* susceptibilities to CQ, lumefantrine (LU), dihydroartemisinin (DHA) and piperaquine (PQ) (Bai *et al*., 2018). A greater than 60%, 50% and 7% frequencies were evidenced in H191Y and S437A, I876V and F1390I polymorphisms respectively in the population (Bai *et al*., 2018). In addition, in Thai-Myanmar border *Pfmrp1* F1390I SNP was considerably linked to ART, MQ and LU in *in vitro* reduced sensitivity test in the population (Veiga *et al*., 2011).

2.6 The Trends of *Plasmodium falciparum* multidrug resistance protein 1 (*Pfmrp1)* polymorphisms and correlation with *in vitro* antimalarial drug sensitivities in Africa

AL and AS-AQ are the most common and currently used ACTs in the treatment of uncomplicated malaria in Africa especially East Africa (Holmgren *et al*., 2007). In Thailand and Angola (West African) isolates a prevalence of 95.3% *Pfmrp1* alleles 191Y and 100% 437A were evidenced to be associated with *in vitro* drug responses to MQ. This was done to investigate if the same polymorphisms from Asia and South America associated to MQ, as well as CQ and QN in *in vitro* responses, would be described in the other regions also (Nogueira, Lopes, Alves, & Rosario, 2008). Another *in vivo* study conducted in East Africa found the most prevalent non-synonymous SNPs in Africa were I876V and K1466R that are largely spread in Asian, African, and Oceanian parasite populations (countries between Indian Ocean and North Pacific Ocean such as Australia), I876V was evidenced to be under selection after LU (Dahlstrom *et al*., 2009). In sSA a study was conducted to assess reliable tools in monitoring anti-malarial resistance, SNPs in *Pfmrp1* gene were validated as ideal molecular markers of monitoring resistance to artemether (AM), artesunate (AS), DHA as well as QN in regards to the combination therapies commonly and currently used in Africa (Apinjoh *et al*., 2019).

The frequency of *Pfmrp1* SNPs compared to other markers has not been vastly studied among African populations as compared to the GMS yet it is also a potential molecular marker for monitoring ACT drug resistance. The need to study it was therefore very critical to help assess drug resistance status in Kenya. sSA is known as a resource-limited zone in several aspects of combating malaria yet it has the highest prevalence of *P.f.* burden. To anticipate the emergence or spread of resistance to anti-malarial drugs in Africa, consistent surveillance of the genotype and phenotype of the parasite was required and above all with ideal tools. This study therefore, facilitated tracking anti-malarial resistance in Kenya by evaluating the frequency of *Pfmrp1*

SNPs associated with anti-malarial resistance and correlation with *in vitro* drug sensitivity patterns between 2008 and 2019 to compare the transition period and post-ACTs trends.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study Area

The study analysed samples collected under the ongoing epidemiology of malaria and drug resistance sensitivity patterns in Kenya. This surveillance study started in 2008 and enrolled individuals from diverse geographical locations in Kenya comprising four of the five major epidemiologic zones of malaria including: the Lake endemic transmission (Kisumu (KDH) and Kombewa (KOM), Semi-arid (Marigat (MGT), seasonal transmission (Malindi (MDH), and highland epidemic transmission, Kisii (KSI) and Kericho (KCH).

Kenya is considered a malaria-risk area due to presence of five different malaria epidemiological zones namely: lake endemic, coast endemic, highland epidemic, seasonal transmission and low-risk zones described in a survey of febrile patients (Githinji *et al*., 2016). This regional variability in malaria burden in Kenya across geographic ecologies presents a challenge in implementing malaria control and case management interventions. Key interventions include; the provision of long-lasting insecticidal nets, indoor residual spraying, intermittent preventive treatment for pregnant women, prompt diagnosis and effective treatment of all malaria cases, the coastal areas near the Indian Ocean and the Lake Victoria region are high-burden and malaria prevalence hovers around 8% and 27% respectively (KMIS, 2015). Kenya launched the new 2009-2017 National Malaria Strategy (NMS) whose case-management mainstay is parasitological testing of all febrile patients across all age groups and areas of malaria endemicity and treatment of only test positive patients with nationally recommended ACT-(AL) (Nyandigisi *et al*., 2011). Figure 3.1 shows the sites where the samples were drawn from (including County and sub-County hospitals).

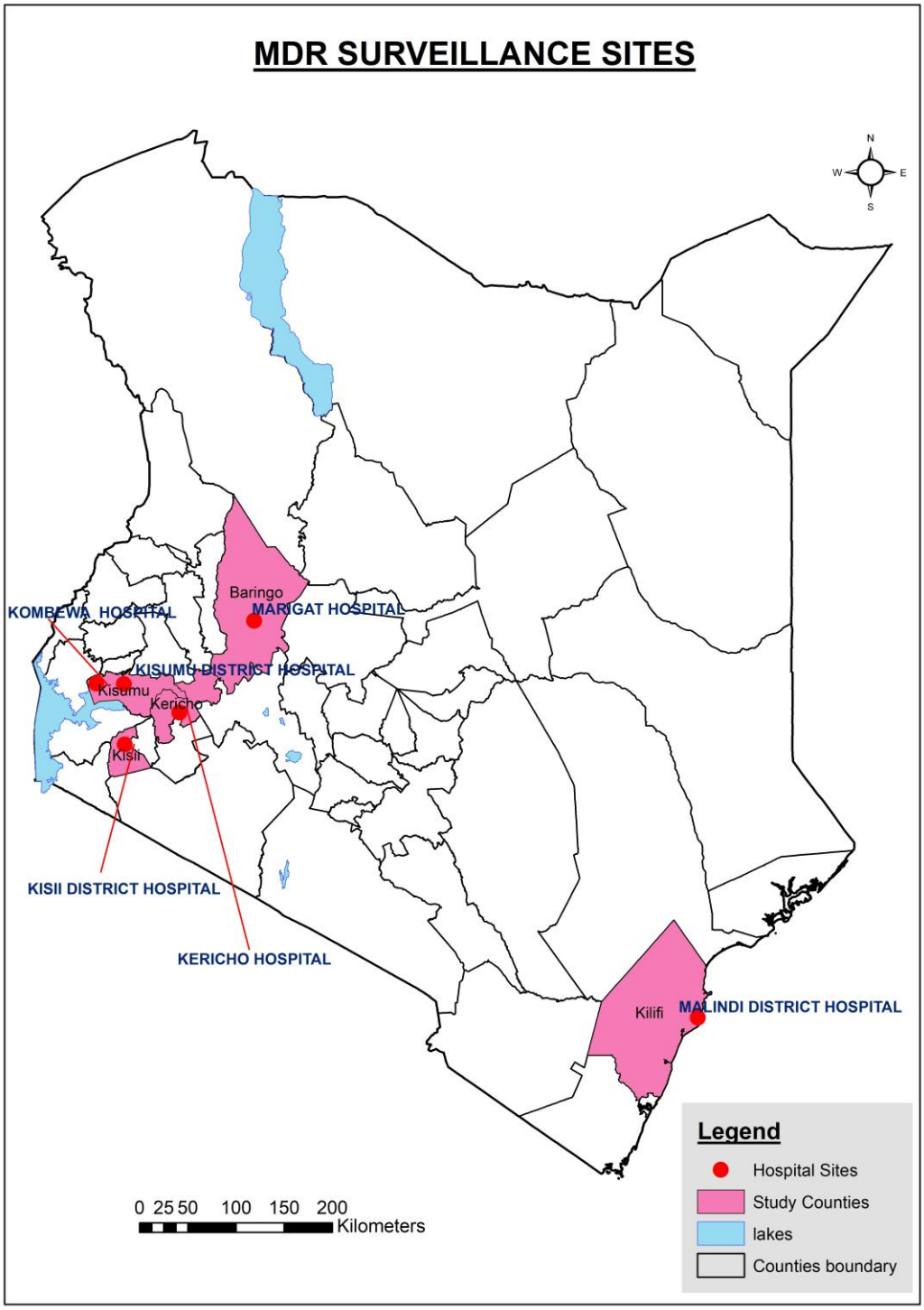


Figure 3.1: Map of Kenya showing the different malaria endemicity zones and locations of various surveillance hospitals adopted from (Muiruri *et al*., 2018).

These sites were chosen to cover different malaria ecological zones of Kenya with different transmission rates. The areas covered include the endemic lake (Kisumu and Kombewa), coastal regions (Malindi), the epidemic-prone highland region (Kisii and Kericho) and the seasonal region (Marigat).

3.2 Study Design

This study was a cross-sectional retrospective survey on archived samples obtained from patients with *P.f.* infections between 2008 and 2019. The samples used were collected at day 0, only one time point of the recruitment day. It was a retrospective study since samples used had been previously collected.

3.2.1 Patient population

The study period focused on 3 different time points between 2008 and 2019. The chosen periods were; 2008-2009, 2013-2014 and 2018-2019. Parasites were categorized in different timepoints to compare the transition period and post-ACTs period, the 2008-2009 time point was categorized as a transition period as described by (Ngalah *et al*., 2015) furthermore, it took some time for ACTs to be effective in Kenya after their implementation in 2006 (Amin *et al*., 2007). The mid-era between post-ACTs and the transition period was categorized between 2013-2014, while Post-ACTs was between 2018-2019. The time-points were given an even three years range between each other.

3.2.2 Recruitment of study participants

The study was part of an ongoing study investigating persons from 6 months and older who were treated for malaria within the previous 14 days and presents with a positive *P.f.* and/or Pan RDT consistent with *P.f.* (both test bands +) or other species (Pan band only). RDTs are within the standard of care for diagnosing *P.f.* malaria in Kenya. For the parent study approximately 100 volunteers per week were enrolled from all the sites which is about 2-3 volunteers per day per site. A subset of 4 individuals per site per month from 6 months of age and older without signs of malaria were also enrolled. This subset served as a source of samples for quality control purposes. Only samples from patients with *P.f.* monoinfections detected by qPCR were selected

and used for the current study that was irrespective of the month of enrollment because of inconsistencies from sites in enrollment.

3.2.3 Screening and enrollment

For the parent study potential volunteers were identified by the attending medical personnel at the study site and then, an informed consent/assent was obtained by the study team staff. The study staff reviewed the inclusion and exclusion criteria to determine eligibility. Questionnaires were used to collect relevant demographic and clinical information. Written informed consent was administered in the participant's language of choice. After that enrolled volunteers had a single 2.5 ml sample of blood drawn under sterile technique at the time they became eligible for enrollment. From this blood, 2 malaria smears, 4 blood spots, and whole blood for parasite culture was prepared. The Pf/Pan RDT was done using the whole blood sample.

3.2.4 Inclusion criteria

Individuals aged six months and above, presenting at outpatient departments with symptoms of malaria and/or testing positive for uncomplicated malaria by rapid diagnostic test (mRDT; Parascreen® (Pan/Pf), Zephyr Biomedicals, Verna Goa, India) were recruited into the study after providing written informed consent or assent.

3.2.5 Exclusion criteria

Volunteers not willing to; complete the questionnaire or participate in the blood draw, consent to have their blood stored for future use and those that are currently detained by the Kenya Government Department of correctional services, also children under age 18 without available parent or legal guardian.

3.3 Sample Size Determination

The sample size for this study was calculated by the Cochran's formula 1963 (Duru *et al*., 2016) taking into consideration the prevalence of malaria in Kenya is 20% as reported by the Kenya Malaria Operational Plan 2019 (USAID, 2019).

The following formula was used:

$$n = \frac{Z^2pq}{e^2}$$

$$n = 246$$

Where:

- Z is the critical value for a 95% confidence level (1.96)
- e is the desired level of precision (margin of error) given as 5%
- p is the (estimated) proportion of the population which has the attribute in question (20%)
- q is 1 – p.

The minimum sample size was 246 however, a total of 300 samples were available for the study. The 300 samples were distributed evenly across the 6 sites that was 20 samples per site per timepoint (3) resulting to 60 samples in KDH, KCH and KSI however, 3 sites as follows; KOM, MGT and MDH lacked samples for the transition period and resulted to 40 samples per site.

3.4 Sample collection and processing

Approximately 0.5 ml from the 2.5 ml blood was collected into a tube containing 1.5ml of transport medium for parasite culture/genetics. Microscopy was used to diagnose malaria in the collected samples. About 200µl whole blood samples that were stored at -80°C collected in EDTA microtainers were used for DNA extraction and eluted in 150µl of elution buffer with

QIAamp DNA Mini Kit protocol (Qiagen, Valencia, CA) as per the manufacturer's instructions. These samples containing genomic DNA were later used for genotyping.

Another set vials of cryopreserved culture samples as described in Appendix 1 from the same study participants that had been stored in liquid nitrogen were thawed to revive samples and re-cultured. Upon culture-adaptation, the field isolates along with reference clones were tested against standard antimalarial drugs. The drug sensitivity assays tested resistance against a range of antimalarial drugs selected as follows; ART, LU, AQ, MQ, QN and CQ.

3.5 Laboratory procedures

3.5.1 Diagnosis of Plasmodium DNA

To confirm presence of parasite DNA a very sensitive quantitative PCR assay specific to the genus was done to detect and quantify *Plasmodium* through amplification of 18S rRNA genes DNA with primers and other components of PCR in the assay described by (Kamau *et al*., 2011). The cycling conditions were initial heating to 96°C for 5 minutes. Then denaturation at 96°C for 10 seconds and primers annealing at 60°C for 30 seconds repeated for 40 cycles. *P.f.* species-specific samples were determined by qPCR using QuantStudio 6 Plex PCR system (Applied Biosystems, Foster City, CA) by primers described in (Perandin *et al*., 2004). Typing was later done using the samples that tested *P.f.* positive only.

3.5.2 Genotyping using the Agena MassARRAY system

The samples were genotyped to determine SNPs at codons: 191, 437, 876 and 1390 in the *P.f.* genome of individuals in the *Pfmrp1* gene using the Agena MassARRAY system (Agena bio-sciences, San Diego, CA, USA). The primers used in the assay have been described by (Yeda *et al*., 2016). For the assay, the PCR mix comprised of 10X PCR buffer, $MgCL_2$, dNTP mix, forward and reverse primers (10µM) (pooled together in groups), Taq DNA polymerase enzyme and dH_20. To each well 4µl of mastermix and 2µl of sample were added for the primary PCR.

The cycling conditions for the MassARRAY first PCR were initial heating to 95°C for 2 minutes. Then denaturation at 95°C for 30 seconds and annealing of the primers at 56°C for 30 seconds and 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. The Shrimp alkaline phosphatase (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. The SAP mix consisted of 10X SAP buffer, SAP enzyme and dH_20. On the primary PCR amplicons plate, to each well 2µl 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 (4µM), iPLEX enzyme and dH_20. Then 2µl 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 and finally at 72°C for 3 minutes and holding at 4°C for infinity. Resin clean up was then done with suspending resin to the sample plate via a dimple plate and later adding 42µl of dH_20 per reaction and centrifuging at 3500 rpm for 5 minutes to let it settle off resin. Samples were rotated for proper mixing for approximately 30 minutes prior the run on the machine.The *P.f.* 3D7 strain was used as a positive control while the malaria negative individuals recruited during the study period were used as negative controls for the assay. 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).

3.5.3 Bioassay/*in vitro* antimalarial drug testing

3.5.3.1 Sample revival

A vial of frozen parasites as described in Appendix 1 was thawed by immersing the cryovial in to a pre-warmed water bath at 37ºC for 2 to 5 minutes while carefully ensuring that the water does not touch the lid of the sample vial. After the sample was fully thawed, the volume of the sample was immediately estimated and denoted as (v) for subsequent assaying. 1/5 volume (1/5v) 12% sodium chloride (NaCl) solution was added dropwise slowly while swirling the tube to mix the sample and the solution to homogeity after every drop. It was then let to stand at room temperature for 5 minutes. 9 volume (9v) NaCl 1.6% solution was subsequently added and mixed gently to homogeneity prior to centrifuging on the (Eppendorf, Hamburg, Germany) at 1500 rpm for 3 minutes. The supernatant was aspirated. To the same vial, 9 volume (9v) 0.9% NaCl 0.2% dextrose solution was added, mixed gently and centrifuged (Eppendorf, Hamburg, Germany) at 1500 rpm for 3 minutes. The supernatant was aspirated and the remaining sample pellet resuspended in 10% tissue culture media, 4.5ml and 0.5 ml washed red blood cells (RBCs) as prepared in Appendix 1, 50% for a 5ml culture. The pellet was re-suspended in 5 ml RBCs suspension in complete media, 5% hematocrit and put into a 25 cm^2 flask to culture adapt as the growth was monitored as described in Appendix 1 for approximately 5 cycles.

3.5.3.2 *In vitro* anti-malarial drug testing using SYBR green 1 technique

The parasitemia of all malarial stages of culture was first determined using microscopy as described in Appendix 1. Culture-adapted samples with parasitemia exceeding 0.3% but less than 1% were tested without lowering parasitemia while those at greater than 1% had their parasitemia lowered to 1% in complete culture medium as prepared in Appendix 1. Hematocrit for both 0.3% to 1% and greater that 1% samples was adjusted to 2%. 100µl malaria-infected erythrocytes were added to each well on a pre-dosed drug plate with ART, LU, AQ, MQ, QN

and CQ. The standard drugs from worldwide antimalarial resistance network (WWARN) were dissolved and a 2 fold serial dilution done across the 95 well plates with 11 different concentrations from the stock consisting of different starting concentrations as shown in Appendix 1. The cultures were then incubated for 72 hours at 37°C in a humidified chamber (Thermo Fisher Scientific, Waltham, Massachussetts), under a gas mixture of 90% nitrogen, 5% oxygen and 5% carbon dioxide for activity responses. After the 72 hours, 100µl lysis buffer containing 1x SYBR Green I was subsequently added to each well and the plates incubated for up to 24 hours at room temperature in the dark to terminate the reaction. Fluorescence was then read on a fluorescence plate reader with excitation and emission wavelength bands centered at 485 and 530 nm, respectively in a Tecan machine (Life Technologies, Waltham, Massachussetts) to determine the IC_{50}s values of the samples. Already established and validated controls for the assay by WHO and WWARN were; W2/DD2 for MQ sensitive parasites while 3D7/D6 for MQ resistance parasites but, for CQ it was a reverse of MQ controls for its sensitive and resistance parasites, F32-ART for ART resistance parasites and F32-CEM for ART sensitive parasites however, 3D7/D6 and W2/DD2 were used as standard controls for AQ, LU and QN as they are yet to have established controls.

3.6 Data Management

Data was entered into a Microsoft Excel 2010 spreadsheet. Mass Array typer version.4.0 was used in SNP genotype calling against the reference 3D7 genome (Chebon *et al*., 2016) to determine wild type, mutant or mixed genotypes. Frequencies were determined as percentages (%) in Microsoft Excel 2010. $IC50_S$ data was categorized in Microsoft Excel 2010 spreadsheet.

3.6.1 Statistical Analysis

Chi-square test for independence was used to determine the differences in frequencies of SNPs in the *Pfmrp1* gene within polymorphisms at codon 876V across time and between regions using R

software v.3.5. Antimalarial drug sensitivity/resistance parasite patterns were determined as median $IC50_S$ (upper and lower interquartile ranges (IQR). The non-parametric tests (1-way ANOVA Kruskal-Wallis with Bonferroni's Multiple Comparison Test) for comparing medians in more than four different groups in Graph pad Prism 5.0 software was used to determine the antimalarial drug sensitivity/resistance pattern analyses across sites and between different timepoints in Kenya. The Mann Whitney U test in Graph pad Prism 5.0 software was used to determine significant differences in the median $IC50_S$ of sensitive parasites versus resistance in Kenyan isolates. To establish the correlation between the polymorphic in the *Pfmrp1* gene and *in vitro* anti-malarial susceptibility profiles for the Kenyan isolates, the non-parametric tests (1-way ANOVA Kruskal-Wallis with Bonferroni's Multiple Comparison Test) in Graph pad Prism 5.0 software was used. All statistical analyses were performed at the 5% significance level and the corresponding 95% Confidence Interval (CI). Critical significance levels was set at $P \leq 0.05$.

3.7 Ethical considerations

Ethical approval to carry out this study was obtained from the Kenya Medical Research Institute (KEMRI), Scientific and Ethics Review Unit (SERU) and Walter Reed Army Institute of Research institutional review board (WRAIR), protocol numbers: KEMRI #3628 (Appendix 2), WRAIR #2454 as well (Appendix 3). Informed written consent was obtained from the study participants and parent or legal guardian of all children participating in the study (Appendix 4). The risks, how risks were minimized, benefits of the study and study data storage were outlined in the consent forms (Appendix 4).

CHAPTER FOUR: RESULTS

4.1 Demographic, clinical and laboratory characteristics of study participants

The study participants all had clinical symptoms associated with malaria with other illnesses that were not coinfections, they were mostly respiratory and urinary tract infections. Males were slightly more than females and most participants were young individuals. Most participants had been infected with malaria before this encounter and the median parasitemia was slightly less than 1.5%/200WBCs considered as low. Table 4.1 shows a summary of demographic, laboratory and clinical characteristics of the study participants.

Table 4.1: Demographic, laboratory and clinical characteristics of study participants

Characteristics	
Gender, n (%)	
Males	156 (52.0)
Females	144 (48.0)
Age (Months)	
Median	72.0
25% IQR	36.0
75% IQR	168.0
Parasitaemia, (%)/200WBC	
Median	1.40
25% IQR	0.20
75% IQR	3.80
Other illnesses, n (%)	
Absence	255 (85.0)
Presence	45 (15.0)
Travel history out of district in the last 2 months, n (%)	
Absence	156 (52.0)
Presence	144 (48.0)
Malaria history n (%)	
Absence	108 (36.0)
Presence	192 (64.0)

Data are presented as the median (lower and upper interquartile ranges) and n (%).
Abbreviations: IQR-Interquatile range

4.2 Frequency of SNPs in the *Pfmrp1* gene

A total of 300 archival field isolates collected from KDH, KOM, KSI, KCH, MDH and MGT between 2008 and 2019 were successfully genotyped for mutations in the four amino acid positions on the *Pfmrp1* gene described as follows; H191Y: H-Histidine and Y-Tyrosine, S437A: S-Serine and A- Alanine, I876V: I-Isoleucine and V-Valine, F1890V: F-Phenylalanine and V-Valine. SNPs were designated as pure (which contains only either wild type or mutant strains) or as mixed (which contained both wild type and mutant alleles based on presence of two major peaks on the MALDI-TOF or spectra as described by (Falzoi, Pira, Lazzari, & Pani, 2013). Table 4.2 shows the overall prevalence of pure wild type and mutants at each codon. Further analysis was performed on the frequency of mutant alleles where all the mixed genotypes were considered as mutants. The prevalence of mutant alleles at *Pfmrp1* codons 191, 437, 876 and 1390 was 3.1% (n=151), 3.3% (n=274), 58.9% (n=275) and 7.1% (n=267) respectively. Codon 876 had the highest frequency of mutations at 58.9% whereas codons 191 and 437 had the least mutations at 3.1% and 3.3% respectively as shown in Table 4.2.

Table 4.2: % Frequency of SNPs in *Pfmrp1* gene in Kenya between 2008 and 2019

% Frequency				
Codons	H191Y	S437A	I876V	F1390I
Wild-type	151 (96.9)	274 (96.7)	275 (41.1)	267 (92.9)
Mutant	151 (3.1)	274 (3.3)	275 (58.9)	267 (7.1)

Data are presented as [n, (%)] for Wild type or Mutant SNPs for frequencies in Kenyan field isolates between 2008 and 2019. (n) represents the number of isolates that were successfully genotyped at each codon.

4.2.1 Frequency of wild-type, mutant and mixed alleles per study site

Table 4.3 shows the frequency of *Pfmrp1* SNPs per study site for the years 2008-2019. SNPs were classified as wild type, mutant or mixed. The frequency of the mutant allele at codon 876 was highest in Marigat and Kisumu parasites with 75% and 47.4% respectively. In addition,

parasites from Malindi and Kericho had a frequency of 15% and 33.3% of the mutant allele at codon 876, respectively. A low frequency of the mutant allele was observed in parasites collected across the study site at codons 191 and 437 with the highest of mutation recorded at 4.8% and 2% frequency respectively. However, though Marigat had a higher frequency of 22.3% and 18.2% for mutations at codons 191 and 437, the number of samples genotyped successfully in codon 191 was low. There were no pure mutant alleles at codon 1390 in samples collected from Kericho, Kombewa and Malindi while the parasites collected from the other study sites had a frequency of less than 6% at codon 1390. The difference in the frequency of the mutations at codon 876 across time, were significantly different only in Kisii and Kericho ($P<0.00001$).

Table 4.3: % Frequency of wild-type, mutant and mixed alleles per study site

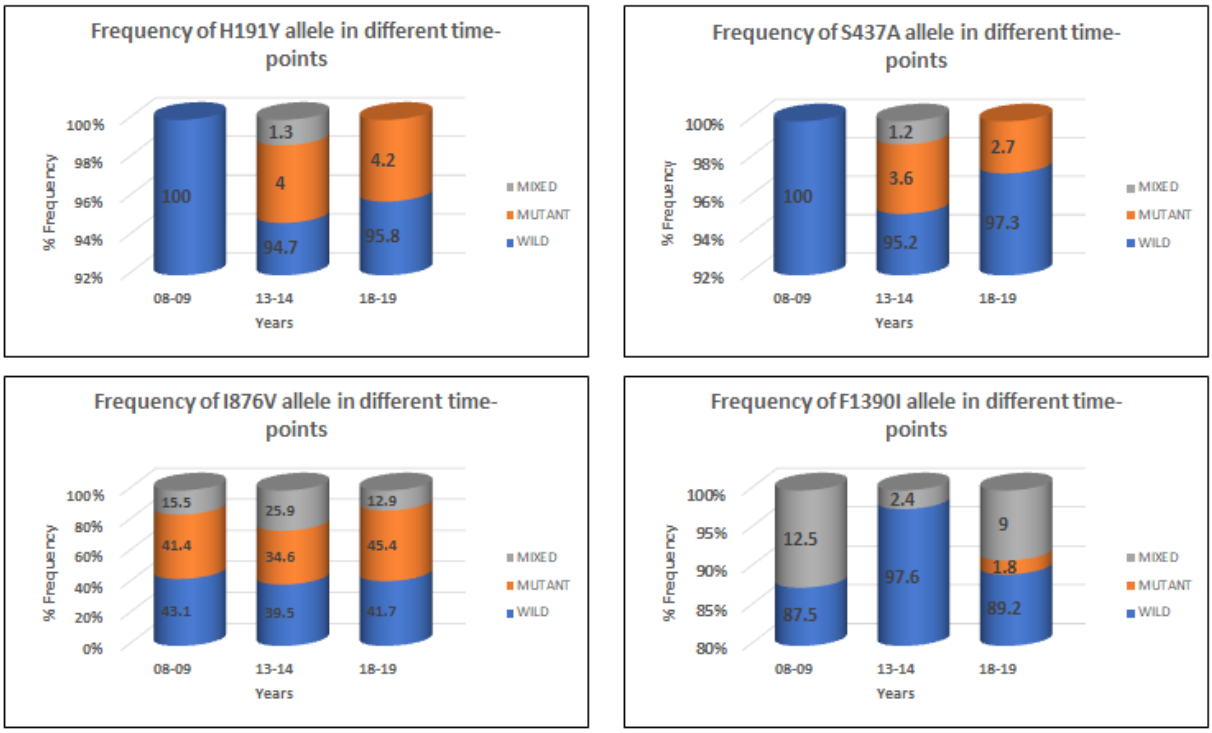
			CODONS		
SITES	**GENOTYPES**	**H191Y**	**S437A**	**I876V**	**F1390I**
		n(%)	n(%)	n(%)	n(%)
	WILD-TYPE	31 (100)	58 (98.3)	19 (33.3)	57 (96.6)
KISUMU	MUTANT	0 (0)	5 (1.7)	27 (47.4)	2 (3.4)
	MIXED	0 (0)	0 (0)	11 (19.3)	0 (0)
	WILD-TYPE	38 (100)	57 (100)	26 (44.1)	56 (98.2)
KISII	MUTANT	0 (0)	0 (0)	23 (39)	1 (1.8)
	MIXED	0 (0)	0 (0)	10 (16.9)	0 (0)
	WILD-TYPE	19 (90.4)	48 (96)	22 (47.9)	48 (96)
KERICHO	MUTANT	1 (4.8)	1 (2)	16 (33.3)	0 (0)
	MIXED	1 (4.8)	1 (2)	9 (18.8)	2 (4)
KOMBEWA	WILD-TYPE	25 (100)	39 (100)	11 (29.7)	39 (97.5)
	MUTANT	0 (0)	0 (0)	17 (46)	0 (0)
	MIXED	0 (0)	0 (0)	9 (24.3)	1 (2.5)
MARIGAT	WILD-TYPE	13 (77.7)	27 (81.8)	8 (25)	25 (66.7)
	MUTANT	4 (22.3)	6 (18.2)	26 (75)	2 (6)
	MIXED	0 (0)	0 (0)	0 (0)	9 (27.3)
MALINDI	WILD-TYPE	19 (100)	36 (100)	26(65)	33 (91.7)
	MUTANT	0 (0)	0 (0)	6 (15)	0 (0)
	MIXED	0 (0)	0 (0)	8 (20)	3 (8.3)

Data are presented as [n, (%)] for Wild type, Mutant or Mixed SNPs for the frequencies in Kenyan field isolates across the study sites between 2008 and 2019. (n) represents the number of isolates that were successfully genotyped at each codon. Over time only Kisii and Kericho had significant differences in the frequencies of the mutations at codon 876, $P<0.00001$ as determined by the chi-square test. Statistical significance was set at $P\leq0.05$.

4.2.2 Frequency of SNPs in *Pfmrp1* gene in Kenya between different timepoints

Figure 4.1 shows the frequency of mutations in the *Pfmrp1* gene at 4 codons: 191, 437, 876 and 1390 for the samples collected from all the 6 sites between 2008 and 2019. An annual increase in the frequency of parasites harboring the mutant in the *Pfmrp1* gene at codon 191 was observed in parasites that circulated in Kenya between 2013 and 2019 with a decline in the wildtype and mixed over the same period. However, the frequency of wild-type alleles kept fluctuating during the study period. Parasites harboring the wildtype had a substantial increase while the mixed alleles at codon 1390 had a reduction in frequency over the 2008-2014 study period with mutant alleles present only between 2018 and 2019.

Figure 4.1: Frequency of SNPs in *Pfmrp1 gene* in Kenya between different timepoints



Data are presented as (%) for Wild type, Mutant or Mixed SNPs for the frequencies in Kenyan field isolates between different timepoints of the study period, each quadrant represents each codon.

4.3 *In vitro* drug susceptible/resistant parasites in Kenyan field isolates

Out of 300 cryopreserved samples that were collected between 2008 and 2019 in Kenya, only a subset of (n=182) parasite samples were successfully analyzed for *in vitro* drug sensitivity tests for 6 selected drugs as follows; CQ, MQ, ART, AQ and LU. Most samples from the low transmission zones when culture adapted were not responding hence were not able to be tested. The 182 samples were distributed across sites as follows; KSI-42, KCH-30, KDH-47, KOM-40, MGT-16 and MDH-7. The parasites were categorized as susceptible versus resistant according to different thresholds per drug. Susceptible parasites are those that had $IC50_S$ within their thresholds while resistant parasites are those that were above the thresholds described as follows; CQ <45ng/ml, MQ<10ng/ml, QN<275ng/ml, these were based on a previous study (Akala *et al*., 2011). The ART, AQ and LU all were at <10ng/ml these thresholds were tested and validated in the lab since their thresholds have not been established. The median $IC50_S$ for the following susceptible versus resistant parasite isolates collected during the study period against the mentioned drugs are shown in Table 4.4.

Table 4.4: IC50s of susceptible/resistant parasites in Kenyan isolates between 2008 and 2019 against selected drugs

	Sensitive parasites						Resistant parasites					
Drugs	**CQ**	**MQ**	**QN**	**ART**	**AQ**	**LU**	**CQ**	**MQ**	**QN**	**ART**	**AQ**	**LU**
Minimum	1.108	0.811	3.122	0.4330	0.1710	1.609	45.84	10.13	375.6	10.93	10.62	10.41
25% Percentile	3.703	2.806	14.26	1.456	1.037	2.208	56.73	11.58	398.8	12.83	13.01	17.36
Median	6.132	4.101	32.68	2.437	2.114	3.894	86.36	16.92	495.2	18.82	14.21	30.60
75% Percentile	11.27	6.623	61.47	4.158	3.339	6.865	116.9	28.82	577.8	30.25	16.68	50.31
Maximum	42.54	9.903	196.3	9.188	8.762	9.679	170.6	71.63	596.3	59.52	18.87	111.3
Samples analyzed (n)	96	118	133	135	151	43	17	37	4	17	10	86
***P*-values**	*P*<0.0001	*P*<0.0001	*P*<0.0001	*P*<0.0001	*P*<0.0001	*P*<0.0001	*P*<0.0001	*P*<0.0001	*P*<0.0001	*P*<0.0001	*P*<0.0001	*P*<0.0001

Column statistics data are presented as the Min, Max, Median, (% lower and upper interquartile ranges) of the sensitive/resistance parasites in Kenyan isolates between 2008 and 2019. (n) represents isolates successfully tested. Results show significant differences in the median $IC50_S$ of sensitive parasites versus resistance ($P<0.0001$). Mann Whitney U test was used. Statistical significance was set at $P \leq 0.05$. Abbreviations: CQ-chloroquine, MQ-mefloquine, QN-quinine, ART-artemisinin, AQ-amodiaquine, LU-lumefantrine.

4.3.1 *In vitro* drug susceptible/resistant parasite patterns across sites

Across sites, parasites were categorized into 3 major epidemiological regions as follows; the endemic (KDH and KOM), epidemic (KSI and KCH) and seasonal (MDH and MGT) regions shown in Figures 4.2-4.4. Among the 3 regions, the seasonal region had no resistant parasites against CQ, QN, ART and AQ while the epidemic region against AQ only. Against CQ the epidemic zone had the least susceptible parasites with median $IC50_S$ of 11.54ng/ml [IQR=6.023-30.29, n=89], while the endemic zone had the most resistant parasites with median $IC50_S$ of 87.29ng/ml [IQR=46.78-473, n=19]. Against MQ the epidemic region had the least susceptible parasites with median $IC50_S$ of 5.116ng/ml [IQR=3.211-7.468, n=119] and the most resistant parasites with median IC_{50S} of 21.71ng/ml [IQR=14-36.85, n=33]. Against QN the epidemic region had the least susceptible parasites with median $IC50_S$ of 58.68ng/ml [IQR=27.66-68.98, n=130] with the highest resistant median $IC50_S$ of 495.2ng/ml [IQR=468.1-522.3, n=4] as well. Against ART, the epidemic region had the least susceptible parasites median $IC50_S$ of 3.232ng/ml [IQR=1.616-5.583, n=134] while the endemic had the highest resistant parasites median $IC50_S$ of 30.25ng/ml [IQR=13.84-38.87, n=16]. Against AQ, the epidemic region had the least susceptible parasites with median IC_{50S} of 2.876ng/ml [IQR=1.493-3.436, n=148] only the endemic region having resistant parasites median $IC50_S$ of 13.97ng/ml [IQR=12.82-15.84, n=9]. Against LU, the epidemic region had the least susceptible parasites with median IC_{50S} of 5.352ng/ml [IQR=2.203-7.8, n=44] and the seasonal with the highest resistant parasites with median IC_{50S} of 34.95ng/ml [IQR=26.32-52.26, n=84].

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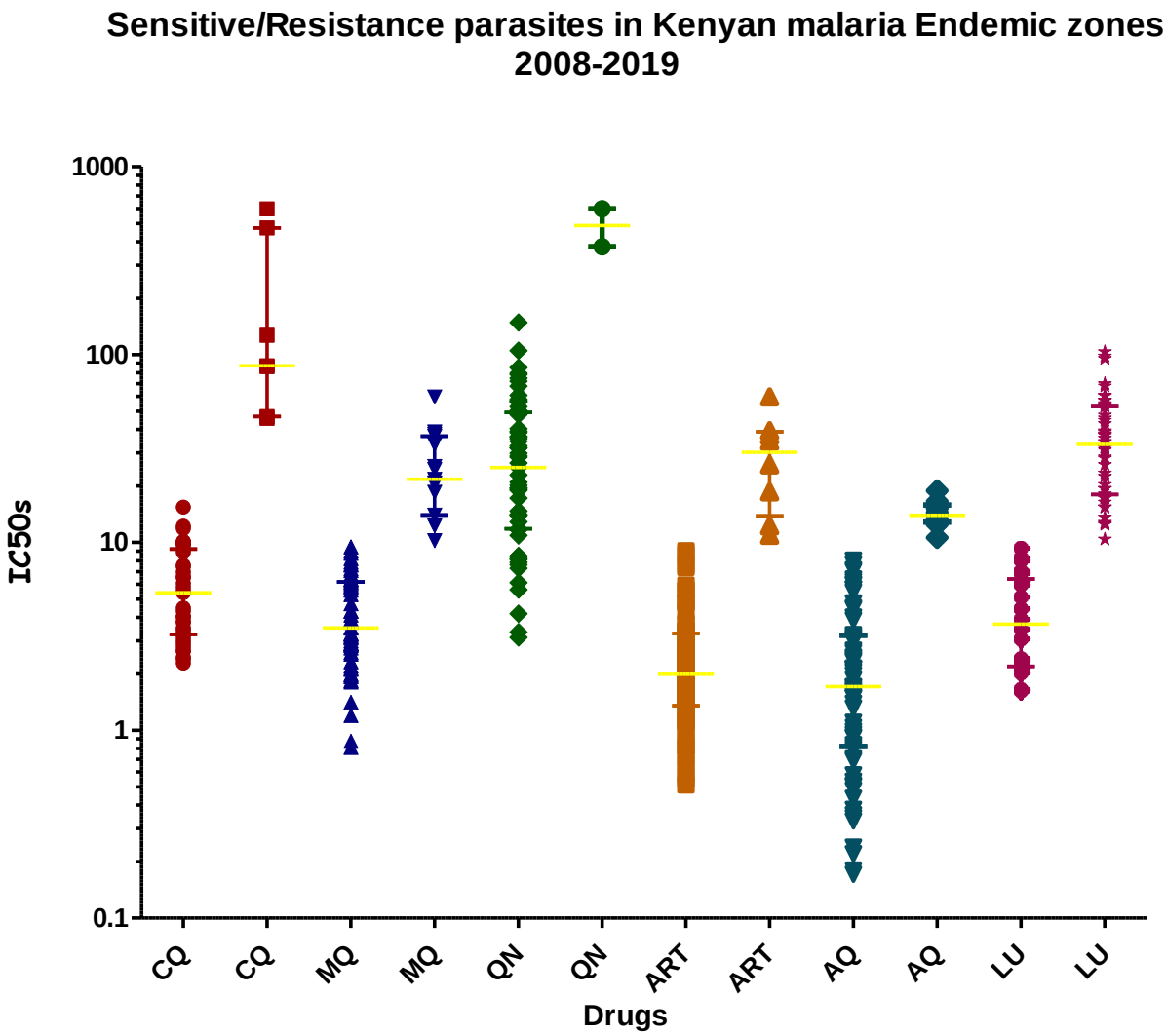
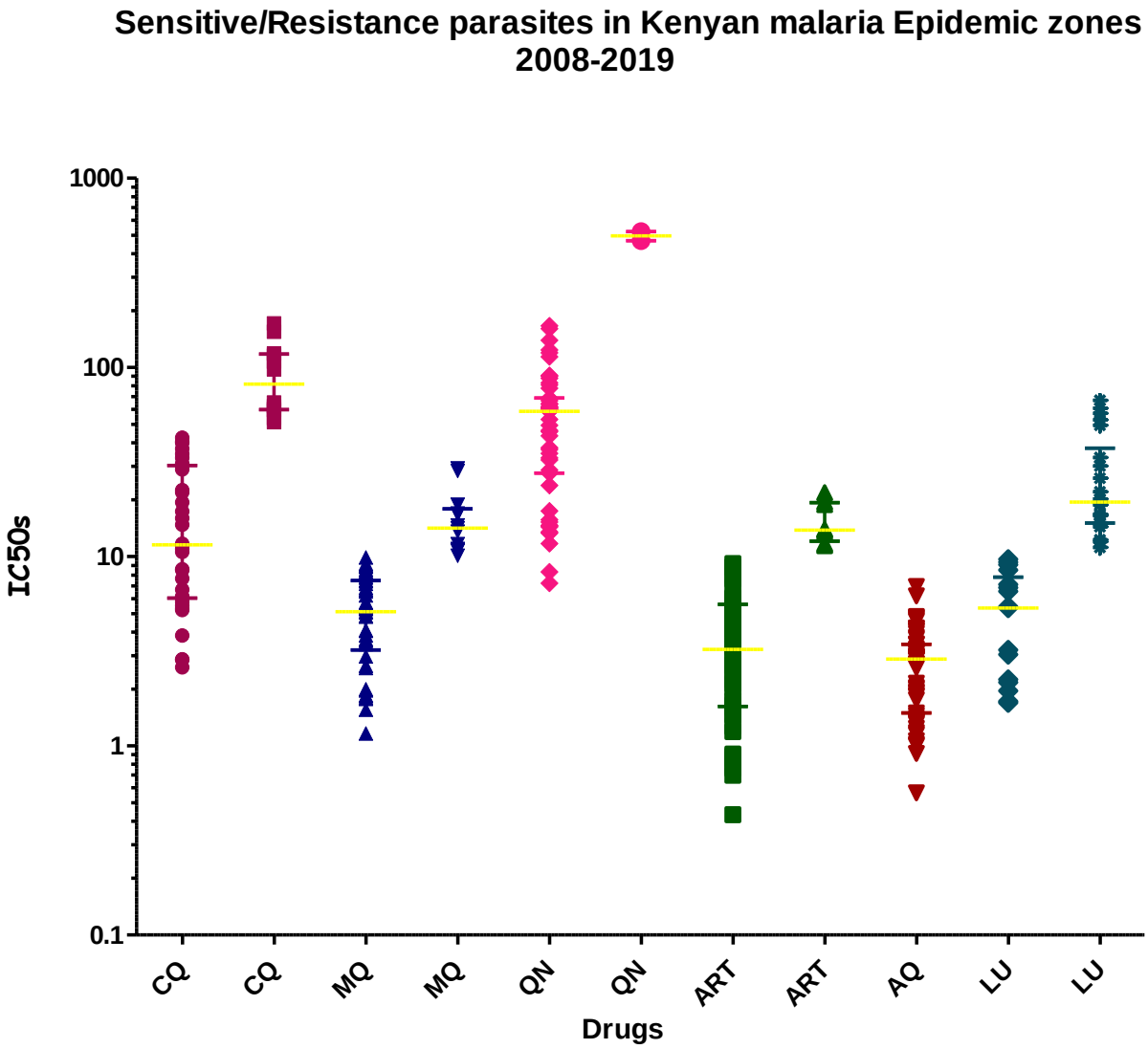
Figure 4.2:Antimalarial drugs susceptible/resistant parasites in Kenyan malaria Endemic zones between 2008 and 2019 against selected drugs



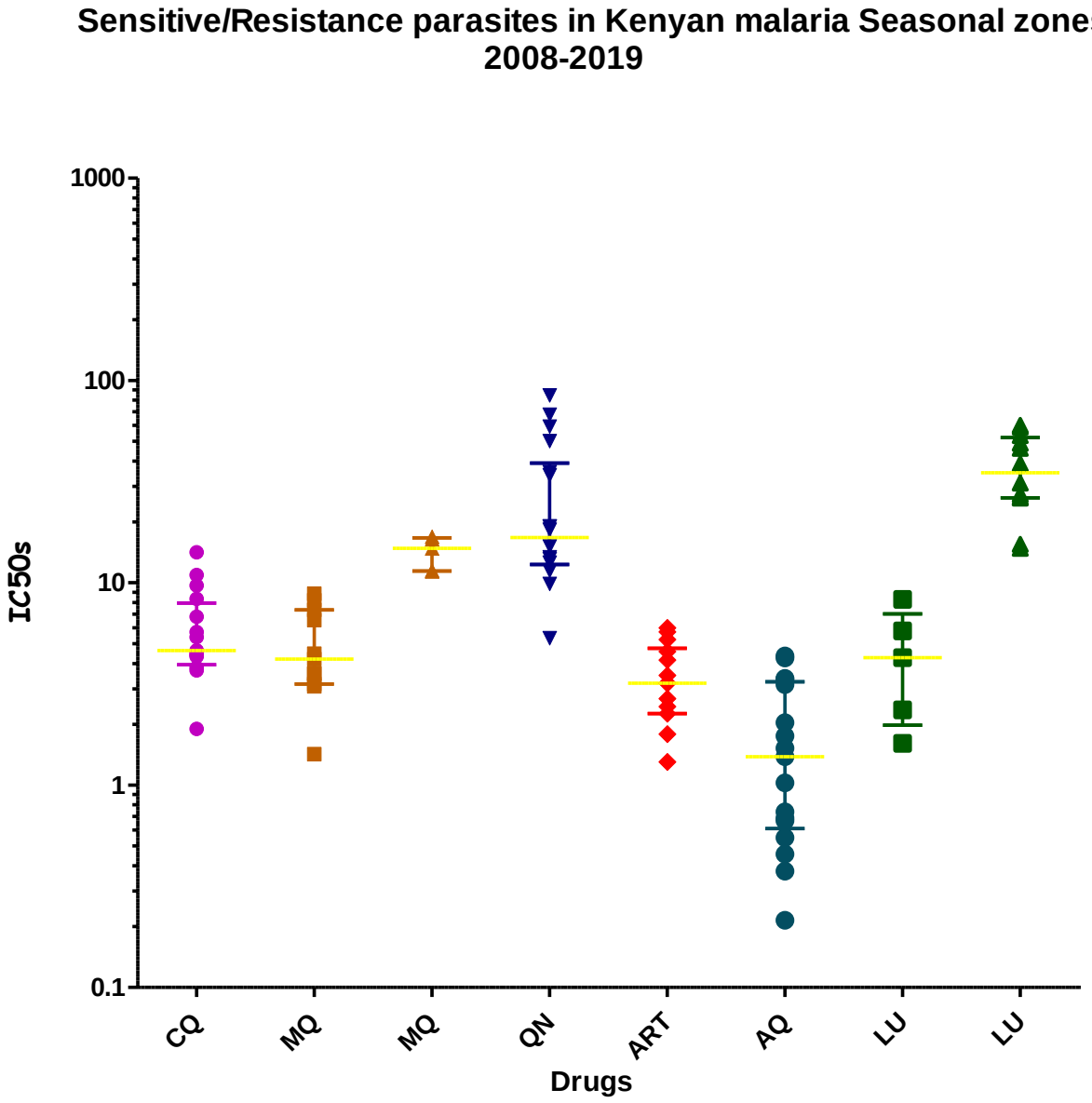
In vitro IC_{50s} pattern for the tested drugs shows the measurements of the sensitive/resistance parasites in the Kenyan endemic malaria zones against selected drugs between 2008 and 2019, (the different colour codes in the figure represent the different drugs tested, yellow represents the medians). Comparisons of the least sensitive versus the most resistant was determined.

Figure 4.3: Antimalarial drugs susceptible/resistant parasites in Kenyan malaria Epidemic zones between 2008 and 2019 against selected drugs



In vitro IC_{50s} pattern for the tested drugs shows the measurements of the sensitive/resistance parasites in the Kenyan epidemic malaria zones against selected drugs between 2008 and 2019, (the different colour codes in the figure represent the different drugs tested, yellow represents the medians). Comparisons of the least sensitive versus the most resistant was determined.

Figure 4.4: Antimalarial drugs of susceptible/resistant parasites in Kenyan malaria Seasonal zones between 2008 and 2019 against selected drugs



In vitro IC_{50s} pattern for the tested drugs shows the measurements of the sensitive/resistance parasites in the Kenyan seasonal malaria zones against selected drugs between 2008 and 2019, (the different colour codes in the figure represent the different drugs tested, yellow represents the medians). Determination of the least sensitive versus the most resistant was observed.

4.3.2 *In vitro* drug susceptible/resistant parasite patterns per drug at different timepoints.

Figures 4.5-4.10 show the patterns of the IC50s of the selected drugs per drug, per time point. Among the drugs chosen, 2 drugs had increased susceptibility over time. These were: AQ and QN. AQ had median IC_{50S} of 2.959ng/ml [IQR=2.453-4.189, n=47], 1.967ng/ml [IQR=1.332-3.243, n=54], and 1.037ng/ml [IQR=0.5031-2.056, n=43]. QN had median IC_{50S} of 60.04ng/ml [IQR=31.85-81.43, n=50], 51.88ng/ml [IQR=33.48-76.04, n=28], and 14.70ng/ml [IQR=8.842-22.43, n=48]. Among the selected drugs only MQ had increased resistant of parasites over time as follows; median IC_{50S} of 16.59ng/ml [IQR=12.13-35.96, n=16], 16.85ng/ml [IQR=11.41-23.79, n=16], and 20.32ng/ml [IQR=12.54-23.12, n=5]. LU however, had contradicting results it had increased susceptibility median IC_{50S} of 7.111ng/ml [IQR=6.562-9.054, n=11], 3.046ng/ml [IQR=2.178-6.175, n=20], and 2.330ng/ml [IQR=2.073-3.675, n=13] with increased resistant median $IC50_S$ of 17.89ng/ml [IQR=15.32-21.89, n=16], 32.48ng/ml [IQR=15.49-52.85, n=31], and 36.02ng/ml [IQR=23.04-53.22, n=32] during the same study period. Results show significant differences at the different time points of every drug (P<0.0001).

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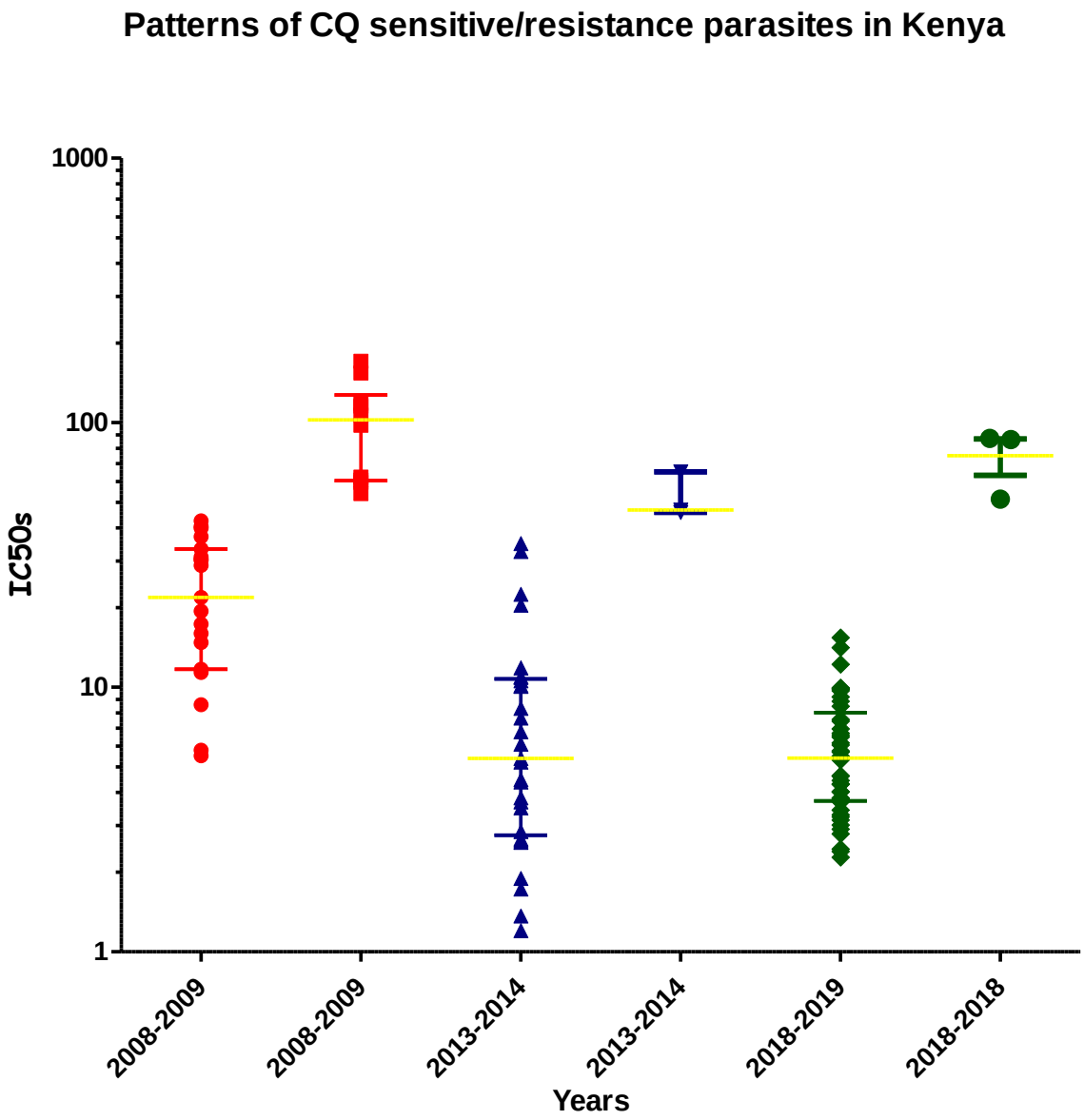
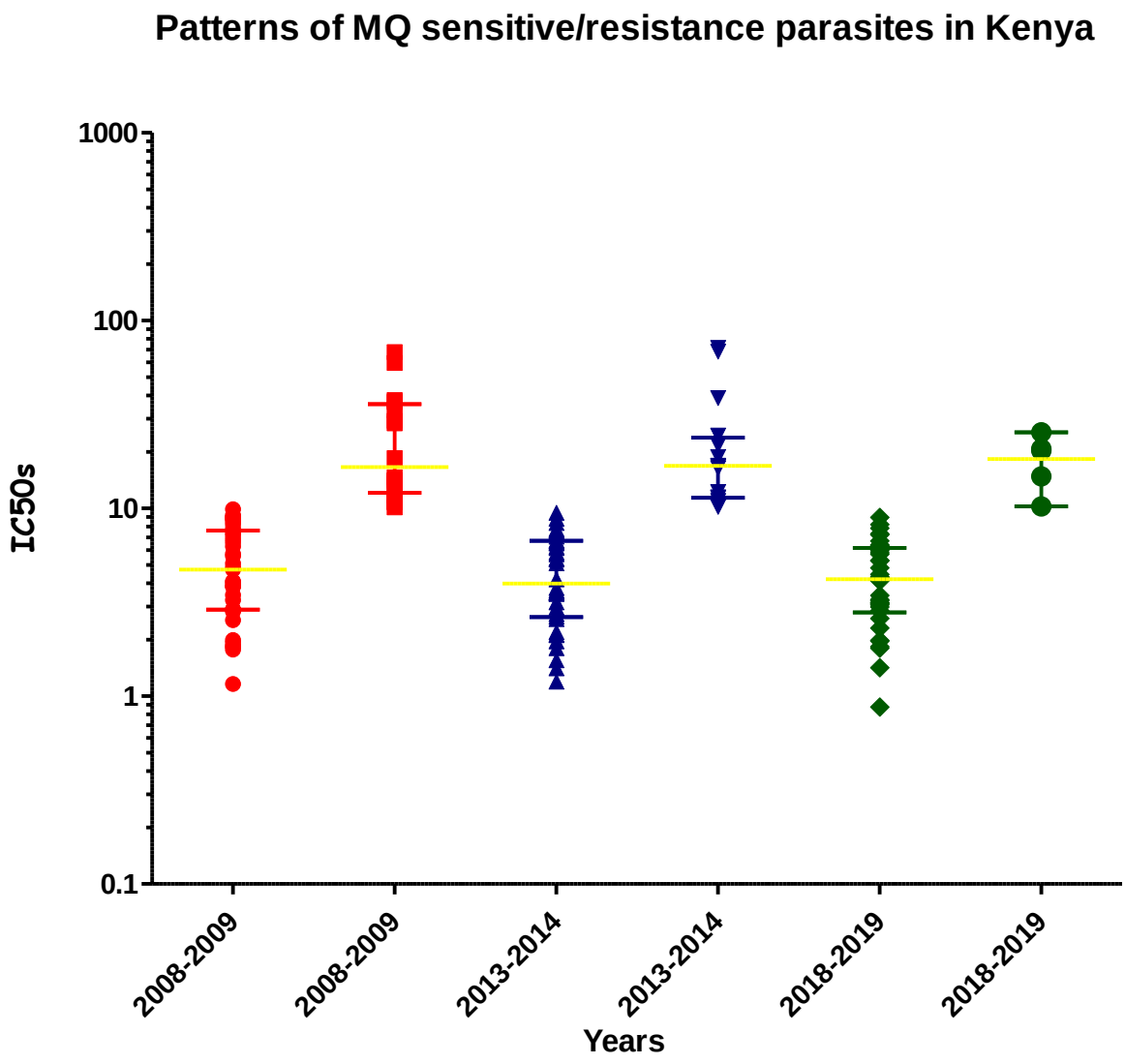
Figure 4.5: Patterns of CQ susceptible/resistant parasites in Kenyan isolates between different timepoints



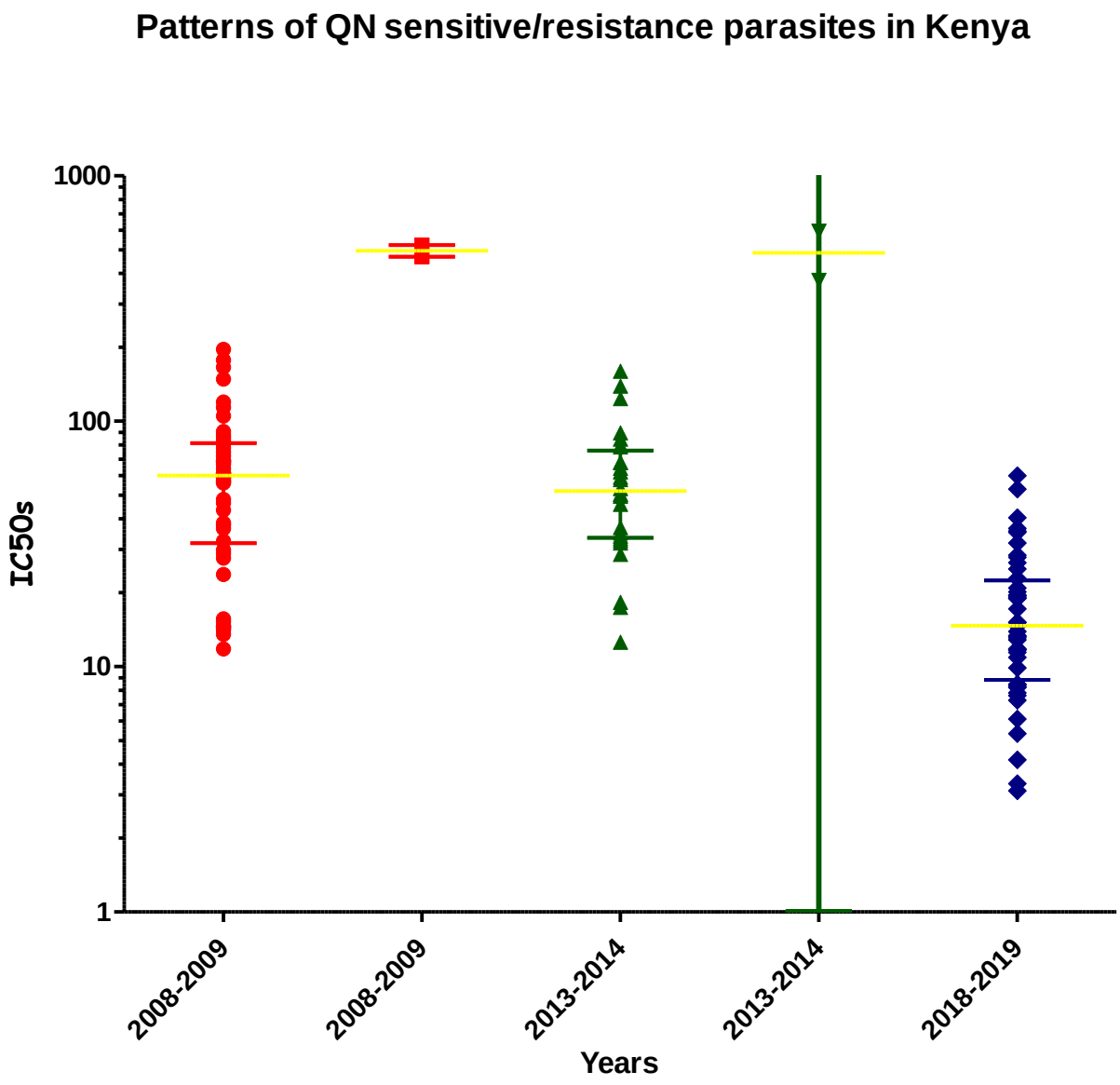
In vitro IC_{50s} pattern for the tested drug shows the measurements of the sensitive/resistance parasites in the Kenyan isolates against CQ between 2008 and 2019, (the different colour codes in the figure represent the different years per timepoint, yellow represents the medians). Results show significant differences at the different time points of every drug ($P<0.0001$). Non-parametric tests (1-way ANOVA Kruskal-Wallis with Bonferroni's Multiple Comparison Test) was used. Statistical significance was set at $P \leq 0.05$. Determination of fluctuating sensitivity/resistance pattern observed over time. Abbreviation: CQ-chloroquine.

Figure 4.6: **Patterns of MQ susceptible/resistant parasites in Kenyan isolates between different timepoints**



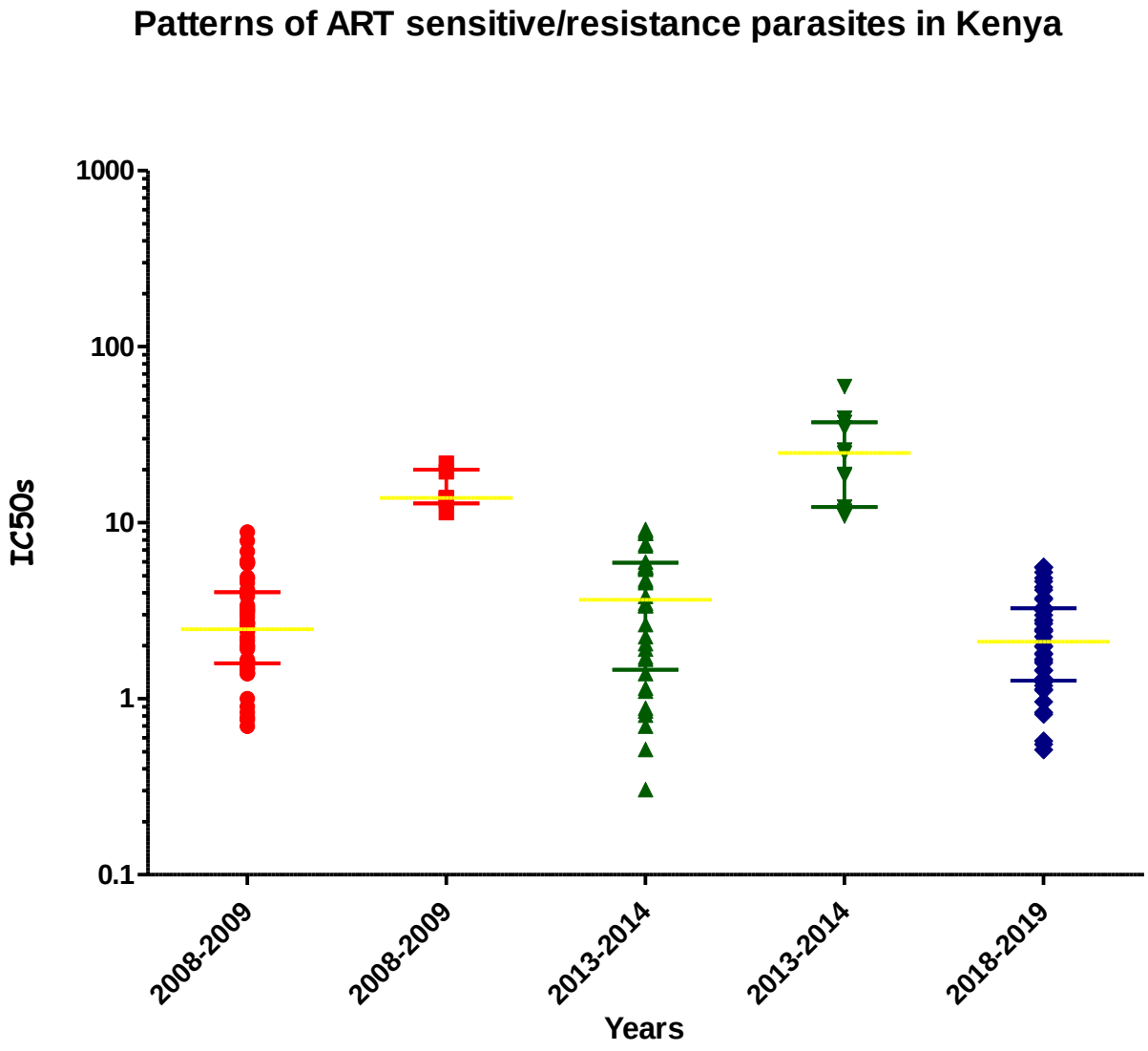
In vitro IC_{50s} pattern for the tested drug shows the measurements of the sensitive/resistance parasites in the Kenyan isolates against MQ between 2008 and 2019, (the different colour codes in the figure represent the different years per timepoint, yellow represents the medians). Results show significant differences at the different time points of every drug ($P<0.0001$). Non-parametric tests (1-way ANOVA Kruskal-Wallis with Bonferroni's Multiple Comparison Test) was used. Statistical significance was set at $P \leq 0.05$. Determination of increased resistance pattern observed over time. Abbreviation: MQ-mefloquine.

Figure 4.7: Patterns of QN susceptible/resistant parasites in Kenyan isolates between different timepoints



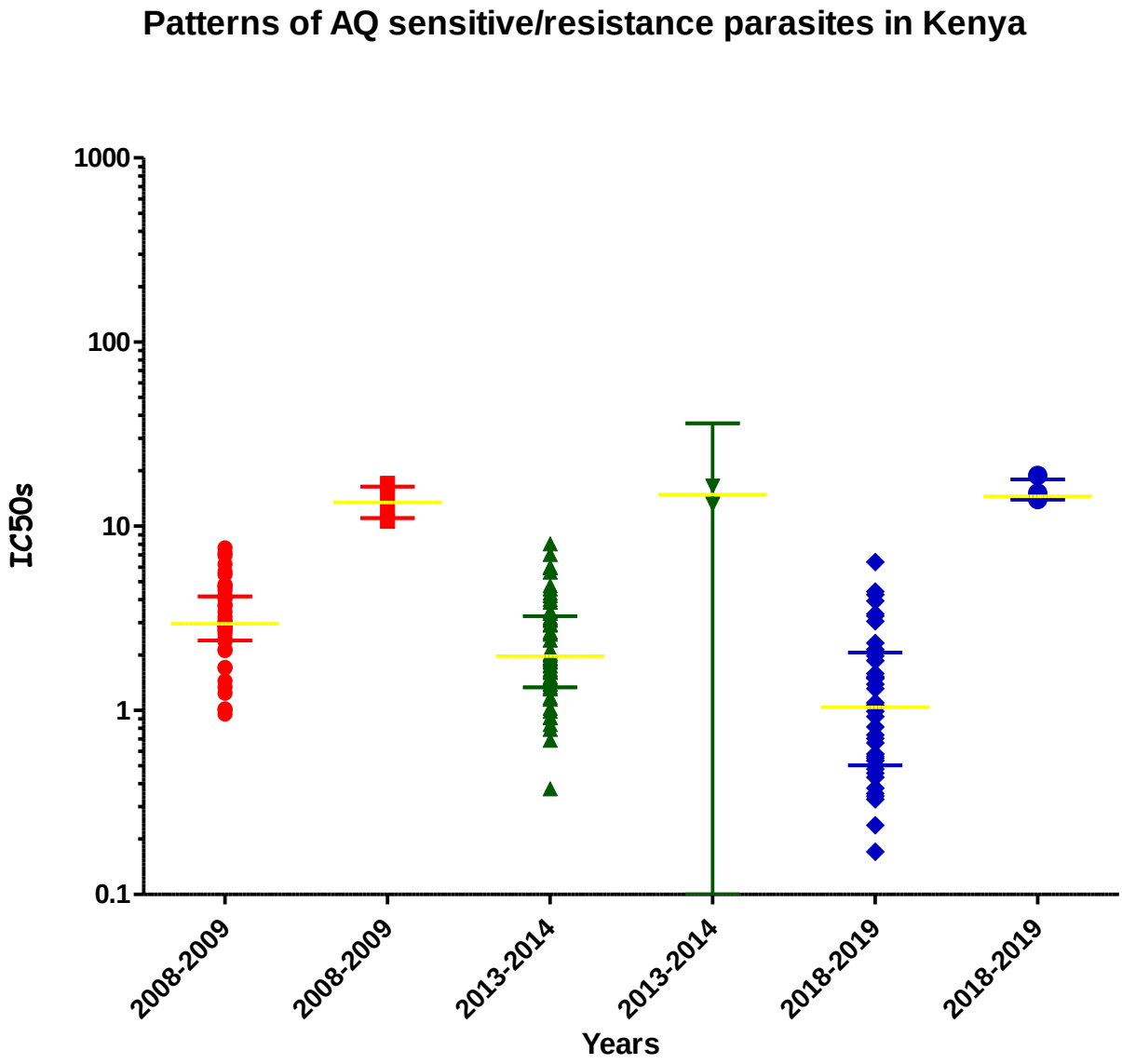
In vitro IC_{50s} pattern for the tested drug shows the measurements of the sensitive/resistance parasites in the Kenyan isolates against QN between 2008 and 2019, (the different colour codes in the figure represent the different years per timepoint, yellow represents the medians). Results show significant differences at the different time points of every drug ($P<0.0001$). Non-parametric tests (1-way ANOVA Kruskal-Wallis with Bonferroni's Multiple Comparison Test) was used. Statistical significance was set at $P\leq0.05$. Determination of increased sensitivity pattern observed over time. Abbreviation: QN-quinine.

Figure 4.8: Patterns of ART susceptible/resistant parasites in Kenyan isolates between different timepoints



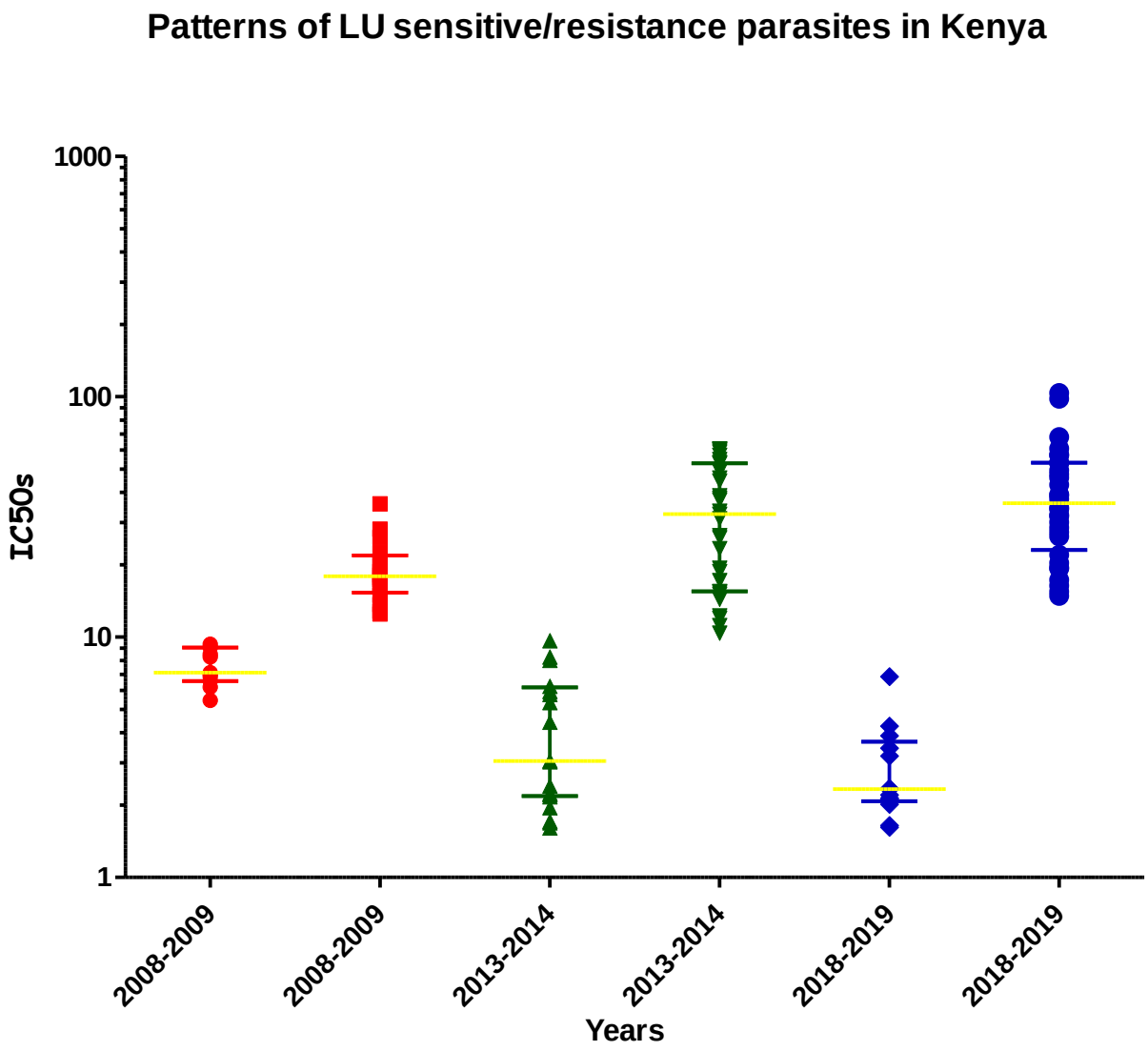
In vitro IC_{50s} pattern for the tested drug shows the measurements of the sensitive/resistance parasites in the Kenyan isolates against ART between 2008 and 2019, (the different colour codes in the figure represent the different years per timepoint, yellow represents the medians. Results show significant differences at the different time points of every drug (P<0.0001). Non-parametric tests (1-way ANOVA Kruskal-Wallis with Bonferroni's Multiple Comparison Test) was used. Statistical significance was set at $P \leq 0.05$. Determination of fluctuating sensitivity/resistance pattern observed over time. Abbreviation: ART-artemisinin.

Figure 4.9: Patterns of AQ susceptible/resistant parasites in Kenyan isolates between different timepoints



In vitro IC_{50s} pattern for the tested drug shows the measurements of the sensitive/resistance parasites in the Kenyan isolates against AQ between 2008 and 2019, (the different colour codes in the figure represent the different years per timepoint, yellow represents the medians). Results show significant differences at the different time points of every drug ($P<0.0001$). Non-parametric tests (1-way ANOVA Kruskal-Wallis with Bonferroni's Multiple Comparison Test) was used. Statistical significance was set at $P\leq0.05$. Determination of increased sensitivity pattern observed over time. Abbreviation: AQ-amodiaquine.

Figure 4.10: Patterns of LU susceptible/resistant parasites in Kenyan isolates between different timepoints

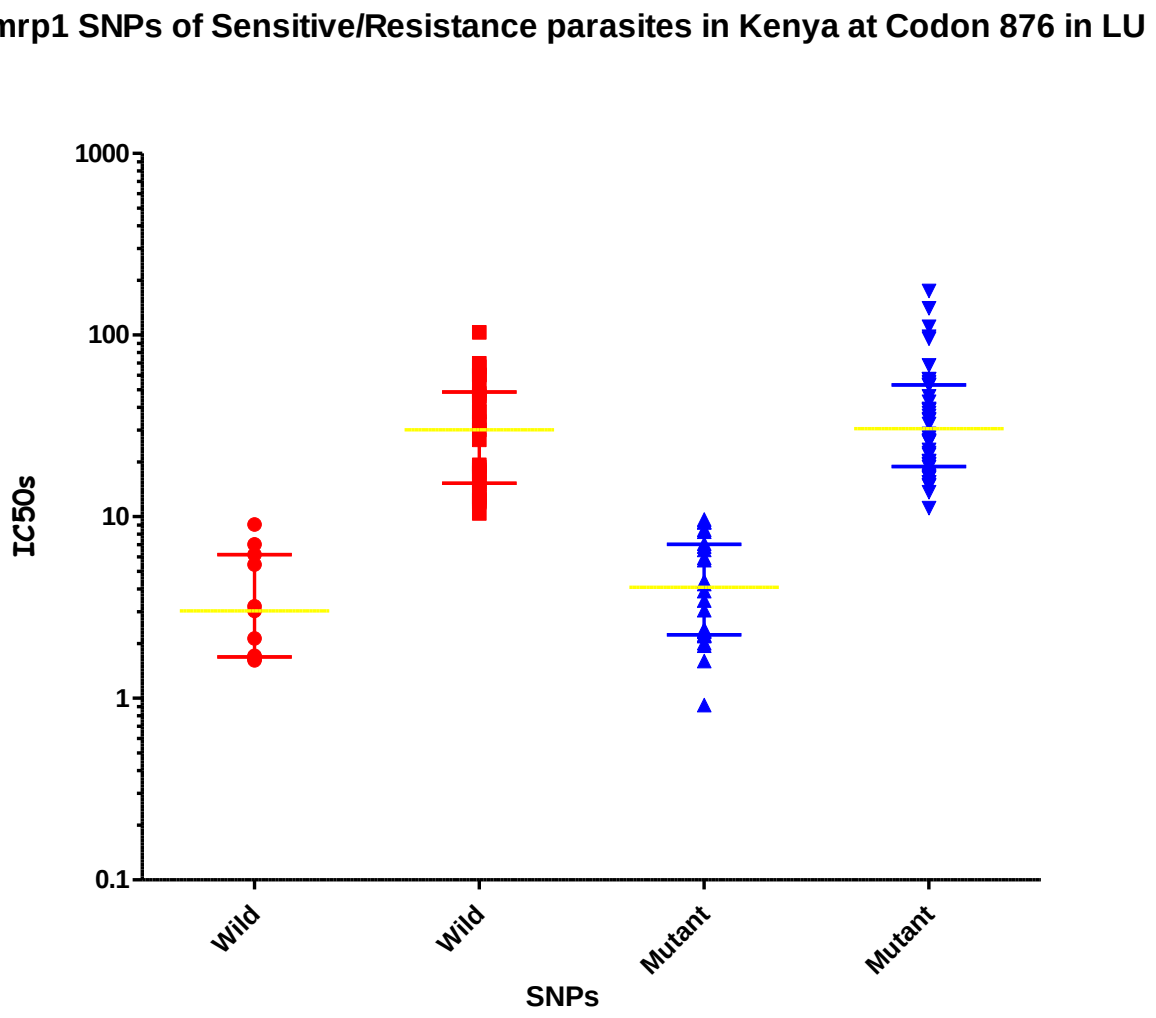


In vitro IC_{50s} pattern for the tested drug shows the measurements of the sensitive/resistance parasites in the Kenyan isolates against LU between 2008 and 2019, (the different colour codes in the figure represent the different years per timepoint, yellow represents the medians). Results show significant differences at the different time points of every drug ($P<0.0001$). Non-parametric tests (1-way ANOVA Kruskal-Wallis with Bonferroni's Multiple Comparison Test) was used. Statistical significance was set at $P\leq0.05$. Determination of increased sensitivity and resistance pattern observed over time. Abbreviation: LU-lumefantrine.

4.4 Correlation of the polymorphic parasites in the *Plasmodium falciparum* multidrug resistance protein 1 gene at codon 876 and *in vitro* anti-malarial susceptibility profiles

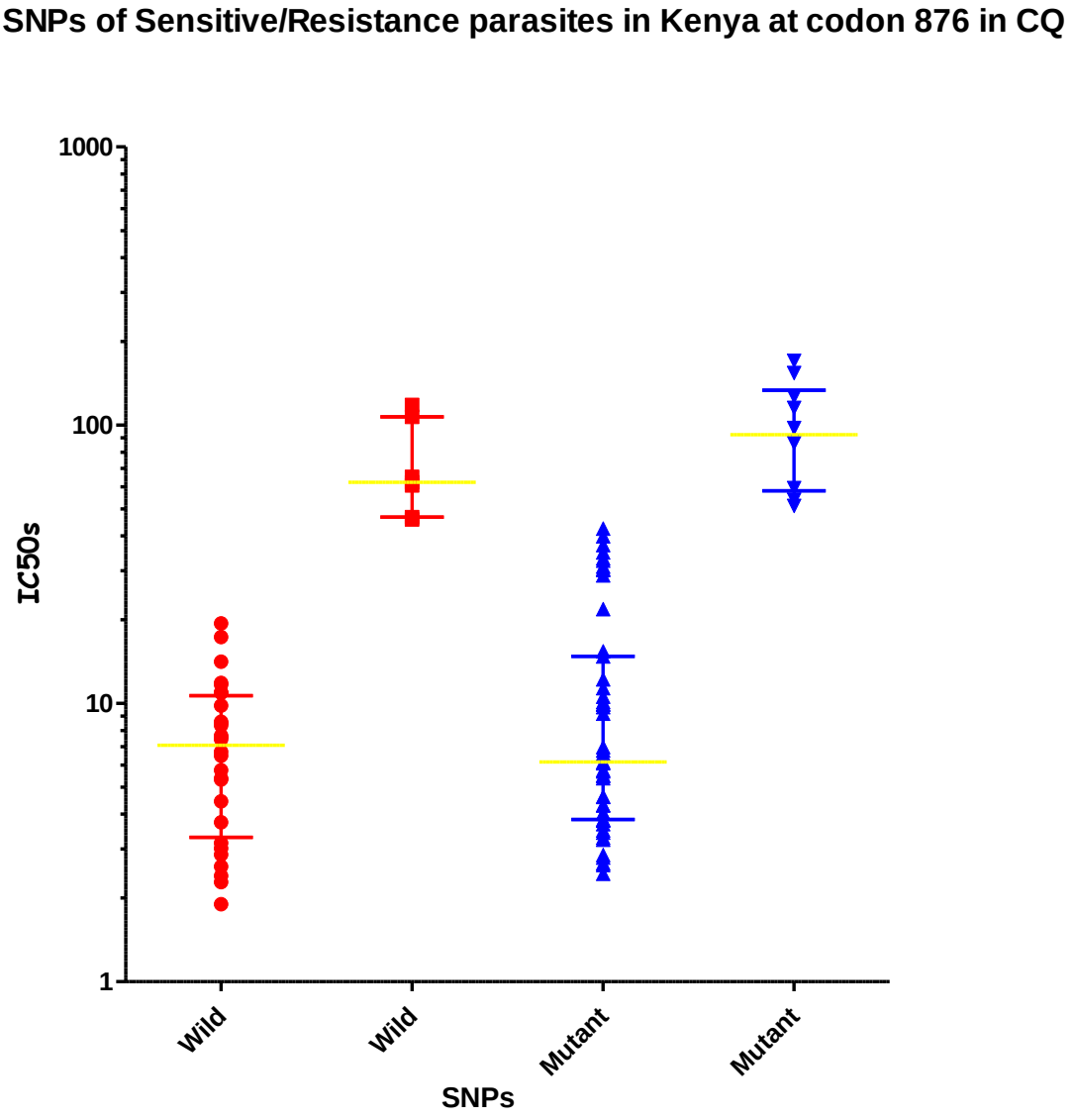
Only polymorphics parasites at codon 876 were comparable with the wild type due to its high frequency compared to the other codons. Figures 4.11-4.16 show the Polymorphic versus wild type SNPs of *Pfmrp1* at codon 876 per drug. Reduced sensitivity was observed in 2 drugs among the selected drugs. LU had a median of 3.034ng/ml [IQR=1.690-6.192, n=11] in wild type parasites and 4.078ng/ml [IQR=2.240-7.050, n=28] in mutant parasites. QN had a median of 35.16ng/ml [IQR=15.24-64.12, n=47] in wild-type parasites and 43.49ng/ml [IQR=15.16-68.05, n=71] in mutant parasites all showing a slight reduction in sensitivity. While ART and CQ showed increased parasite resistance as follows; ART had a median of 13.39ng/ml [IQR=11.41-18.82, n=7] in wild-type parasites and 20.60ng/ml [IQR=13.80-32.44, n=8] in mutant parasites. CQ had a median of 62.35ng/ml [IQR=46.78-107.1, n=7] in wild-type parasites and 92.51ng/ml [IQR=58.09-133.7, n=10] in mutant parasites. Results show significant differences in polymorphic parasites versus wild-type in every drug ($P<0.0001$).

Figure 4.11: Correlation of polymorphic IC_{50s} versus wild type at codon 876 in LU



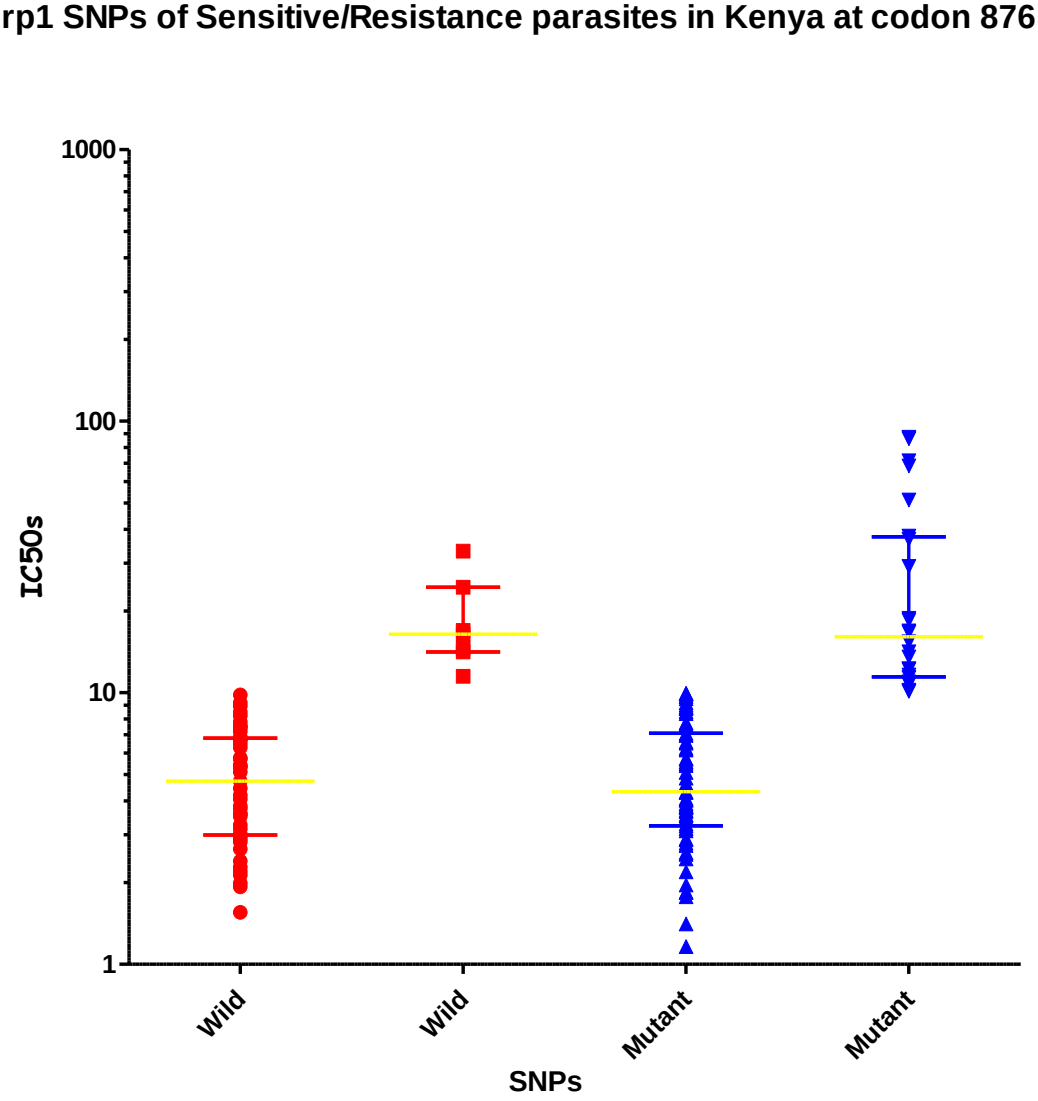
In vitro IC_{50s} pattern for the tested drug shows the correlation of polymorphic IC_{50s} versus wild type at codon 876 in the Kenyan sensitive/resistance isolates against LU between 2008 and 2019, (the different colour codes in the figure represent the different SNPs (Wild/Mutant), yellow represents the medians). Results show significant differences in polymorphic parasites versus resistance of every drug (P<0.0001). Non-parametric tests (1-way ANOVA Kruskal-Wallis with Bonferroni's Multiple Comparison Test) was used. Statistical significance was set at $P \leq 0.05$. Determination of increased or decreased sensitivity/resistance observed. Abbreviations: LU-lumefantrine, SNPs-single nucleotide polymorphisms.

Figure 4.12: Correlation of polymorphic IC_{50s} versus wild type at codon 876 in CQ



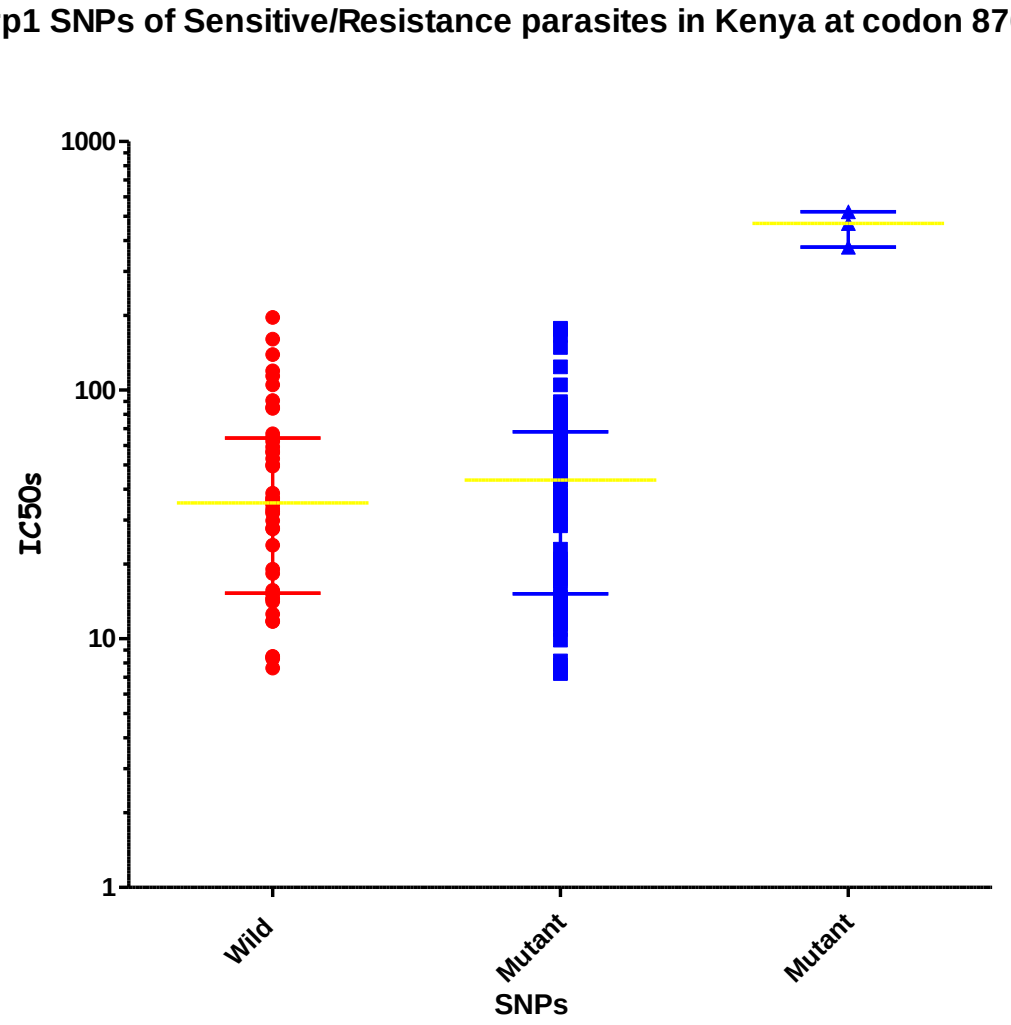
In vitro IC_{50s} pattern for the tested drug shows the correlation of polymorphic IC_{50s} versus wild type at codon 876 in the Kenyan sensitive/resistance isolates against CQ between 2008 and 2019, (the different colour codes in the figure represent the different SNPs (Wild/Mutant), yellow represents the medians). Results show significant differences in polymorphic parasites versus resistance of every drug ($P<0.0001$). Non-parametric tests (1-way ANOVA Kruskal-Wallis with Bonferroni's Multiple Comparison Test) was used. Statistical significance was set at $P\leq0.05$. Determination of increased or decreased sensitivity/resistance observed. Abbreviations: CQ-chloroquine, SNPs-single nucleotide polymorphisms.

Figure 4.13: Correlation of polymorphic IC_{50s} versus wild type at codon 876 in MQ



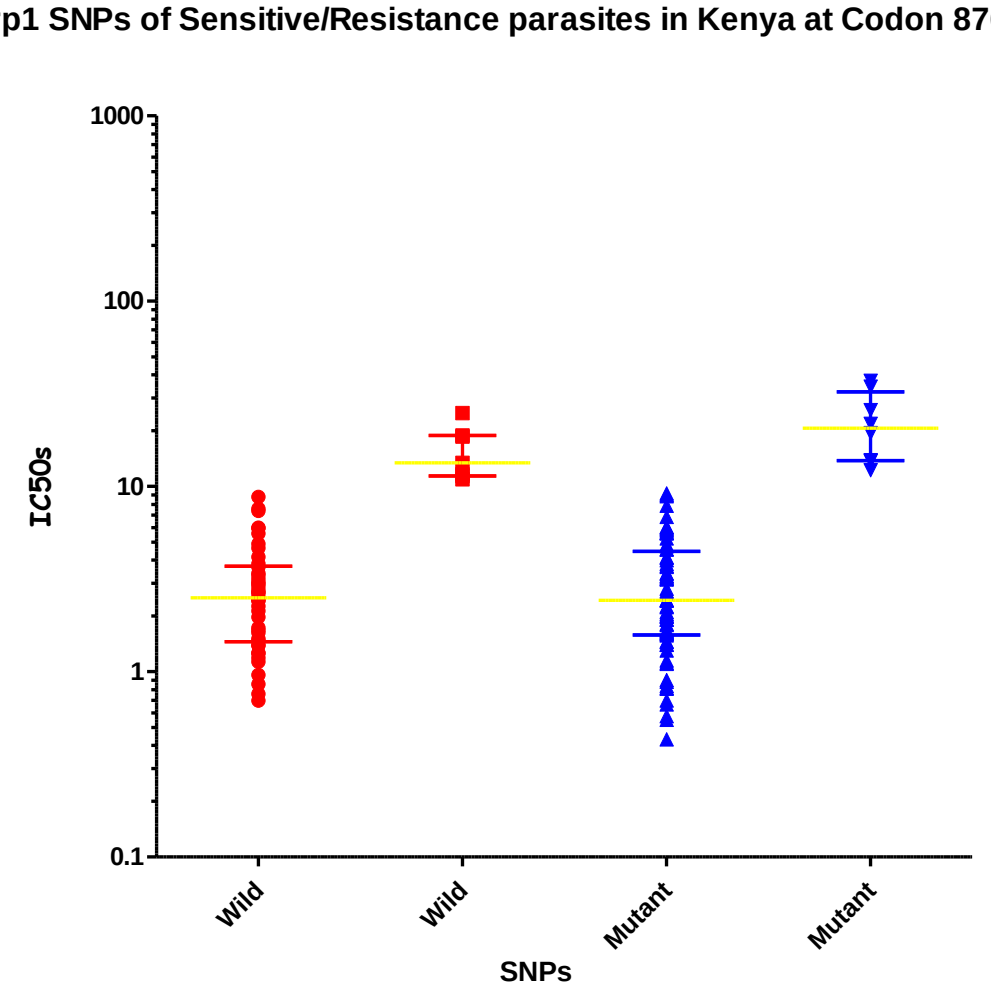
In vitro IC_{50s} pattern for the tested drug shows the correlation of polymorphic IC_{50s} versus wild type at codon 876 in the Kenyan sensitive/resistance isolates against MQ between 2008 and 2019, (the different colour codes in the figure represent the different SNPs (Wild/Mutant), yellow represents the medians). Results show significant differences in polymorphic parasites versus resistance of every drug ($P<0.0001$). Non-parametric tests (1-way ANOVA Kruskal-Wallis with Bonferroni's Multiple Comparison Test) was used. Statistical significance was set at $P\leq0.05$. Determination of increased or decreased sensitivity/resistance observed. Abbreviations: MQ-mefloquine, SNPs-single nucleotide polymorphisms.

Figure 4.14: Correlation of polymorphic IC_{50s} versus wild type at codon 876 in QN



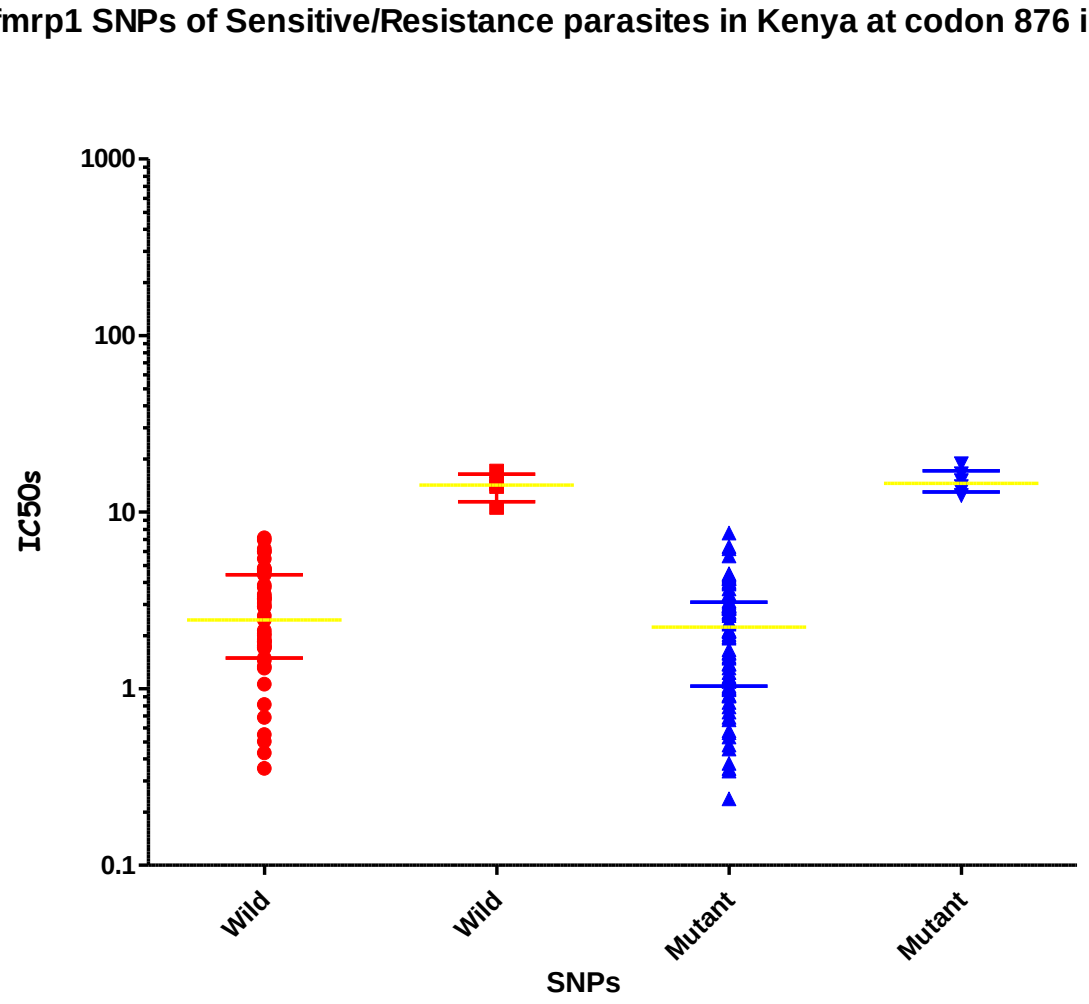
In vitro IC_{50s} pattern for the tested drug shows the correlation of polymorphic IC_{50s} versus wild type at codon 876 in the Kenyan sensitive/resistance isolates against QN between 2008 and 2019, (the different colour codes in the figure represent the different SNPs (Wild/Mutant), yellow represents the medians). Results show significant differences in polymorphic parasites versus resistance of every drug ($P<0.0001$). Non-parametric tests (1-way ANOVA Kruskal-Wallis with Bonferroni's Multiple Comparison Test) was used. Statistical significance was set at $P\leq0.05$. Determination of increased or decreased sensitivity/resistance observed. Abbreviations: QN-quinine, SNPs-single nucleotide polymorphisms.

Figure 4.15: Correlation of polymorphic IC_{50s} versus wild type at codon 876 in ART



In vitro IC_{50s} pattern for the tested drug shows the correlation of polymorphic IC_{50s} versus wild type at codon 876 in the Kenyan sensitive/resistance isolates against ART between 2008 and 2019, (the different colour codes in the figure represent the different SNPs (Wild/Mutant), yellow represents the medians. Results show significant differences in polymorphic parasites versus resistance of every drug ($P<0.0001$). Non-parametric tests (1-way ANOVA Kruskal-Wallis with Bonferroni's Multiple Comparison Test) was used. Statistical significance was set at $P\leq0.05$. Determination of increased or decreased sensitivity/resistance observed. Abbreviations: ART-artemisinin, SNPs-single nucleotide polymorphisms.

Figure 4.16: Correlation of polymorphic IC_{50s} versus wild type at codon 876 in AQ



In vitro IC_{50s} pattern for the tested drug shows the correlation of polymorphic IC_{50s} versus wild type at codon 876 in the Kenyan sensitive/resistance isolates against AQ between 2008 and 2019, (the different colour codes in the figure represent the different SNPs (Wild/Mutant), yellow represents the medians). Results show significant differences in polymorphic parasites versus resistance of every drug ($P<0.0001$). Non-parametric tests (1-way ANOVA Kruskal-Wallis with Bonferroni's Multiple Comparison Test) was used. Statistical significance was set at $P\leq0.05$. Determination of increased or decreased sensitivity/resistance observed. Abbreviations: AQ-amodiaquine, SNPs-single nucleotide polymorphisms.

CHAPTER FIVE: DISCUSSION

5.1 Frequency of SNPs in *Pfmrp1* gene

The current study's results reported a frequency of 58.9% and 7.1% for polymorphisms at I876V and F1390I respectively, during the six year observation period between 2008 and 2019 in Kenya. The previously reported mutations, I876V and F1390I that were found to be associated with *in vivo* ACT response and *in vitro* susceptibility (Dahlström *et al*., 2009; Veiga *et al*., 2011) were also identified in Kenyan isolates. Similarly, both these two mutations have been found in the present study with high frequency distribution for 876V (58.9%) and low for 1390I (7.1%) as previously reported in Thai-Myanmar border population (Veiga *et al*., 2011). Another study previously conducted in three townships of Myanmar population reported a frequency of 59.8%, and 8.5% for 876V and 1390I respectively, which were similar with the present study. The high frequency of 876V mutations observed similarly in both populations might be due to AL selection which led to an increased prevalence of I876V in recrudescent infections in the account of previous studies in several populations (Achieng *et al*., 2015; Gupta *et al*., 2014b; Humphreys *et al*., 2007).

The results of the present study for 191Y and 437A mutations reported frequencies of 3.1% and 3.3% respectively during the study period in Kenya. However, high frequencies of 65.9% and 65.9% respectively, were reported in the Myanmar populations (Zhao *et al*., 2019), the present study reported very different results with the Myanmar population. The high-level of the frequencies of the CQ associated mutations reported in the Myanmar population compared to the current study in Kenyan population could have resulted from continued CQ use for treating *P.v.* infections in the region in spite of, the withdrawal of CQ from treating *P.f.* malaria globally in the endemic regions (Hao *et al*., 2013), most of the SEA parasites might have been selected more since, CQ was shown to be resistance against *P.v* which is more prevalent in their populations compared to East Africa (Menard & Dondorp, 2017a) which habours more of *P.f.*

Based on a previous study AL has been shown to select for the CQ-sensitive parasites (Sisowath *et al*., 2009). The discontinued use of CQ in malaria endemic settings globally after its resistance, has shown evidence of the re-emergence of CQ sensitive parasites previously reported in a Ugandan population, that postulates its possible re-use for malaria management again in future (Balikagala *et al*., 2020). The switch in the treatment regime globally in malaria endemic regions from CQ to AL, corroborates the low frequencies reported in the CQ associated mutations, 191 and 437 in the Kenyan population where *P.f.* CQ-resistant parasites are now reduced.

The results of the current study done in Kenya of a six year observation period between 2008 and 2019 reported frequencies of the polymorphisms as 191Y (3.1%), 437A (3.3%), 876V (58.9%) and 1390I (7.1%). A previous study reported low frequencies of *Pfmrp1* SNPs in Africa compared to Asia and Oceania (Dahlström *et al*., 2009), another study conducted in the Iranian population reported much higher frequencies of 191Y (76.5%), 437A (69.5%), 876V (64.5%) and 1390I (17%) polymorphisms (Pirahmadi *et al*., 2013) reporting similar results with the previous study. The current study corroborates the results of the previous studies based on the much lower frequencies observed in the Kenyan population. This could be due to the different transmission intensities in Africa compared to Oceania and Asia, Kenya is one of the countries in sSA where transmission is highest thus the spread of resistance is not highly favoured compared to the other regions and might not have been adversely affected as much, this is backed by the implication that low, rather than high transmission would favor the spread of drug resistance and high-levels of clinical immunity create a natural ecological refuge for drug-sensitive parasites (Artzy-Randrup, Alonso, & Pascual, 2010). Furthermore, the current study adds knowledge on the frequency of the *Pfmrp1* gene SNPs in Kenya where they were unknown. Nevertheless, the

frequency of these SNPs are already established and well documented in the GMS region as compared to Kenya and East Africa at large where not much has been explored.

5.2 *In vitro* antimalarial drugs susceptibility/resistant parasite patterns

The results of the present study conducted in Kenya between 2008 and 2019 observed LU exhibited a high number of resistant parasites (86 resistant against 43 susceptible parasites) and MQ an intermediate (37 resistant against 118 susceptible parasites) in their responses when compared. Previous studies done in Kenya and Cameroon reported that *in vitro* cross-resistance was largely expected between LU and MQ since they showed a positive correlation, which may be attributed to increased use of AL causing resistance to both drugs, (Basco, Jean, & Pascal, 1998; Eyase *et al*., 2013). The present study was in agreement with these results since LU exhibited a high number of parasites and MQ an intermediate against the isolates analysed, they had no significant differences $P<0.0001$ as well . These observations may be due to the fact that LU might modulate the parasite to institute resistance against MQ due to the physicochemical similarities in the two drugs since they belong to the same class of aminoalcohols. Morover, the underlying reasons for the potential *in vitro* cross-resistance between artemisinin derivatives and aminoalcohols have not been clarified. This previous study thus suggest the mechanisms of action and intraerythrocytic transport processes of these drugs need to be demonstrated for a clearer understanding of the parallel responses among these drugs (Basco *et al*., 1998).

The results of the present study conducted in Kenya between 2008 and 2019 reported CQ exhibited only 17 resistant parasites against 96 susceptible isolates analysed when compared. Previous studies done in Kenya, Malawi and China reported a reduction of CQ resistance parasites in the populations after its withdrawal (Mwai *et al*., 2009; Wang *et al*., 2005). The current study also reported CQ exhibited a few number of resistant parasites against it when compared to susceptible isolates in the Kenyan population corroborating the results of the

previous studies, this may be due to the decrease in CQ drug pressure after a policy change to AL therapy in 2006 which could have facilitated the reduction of the CQ drug pressure, resulting in the reversal of CQ resistance-associated genotypes to the more sensitive genotypes.

In the present study against the selected drugs the most prevalent resistant parasites was observed in the endemic zone (KDH and KOM) compared to the seasonal (MDH and MGT) and highland regions (KSI and KCH). A previous study reported resistance to CQ and SP monotherapy was constantly higher where transmission was extreme (Talisuna, Okello, Erhart, Coosemans, & D'Alessandro, 2007). In the current study, against the selected drugs the most prevalent resistance was observed in the endemic zone compared to the seasonal and highland regions. These observations may be due to the high transmission intensity which is related to high resistance. Furthermore, drug pressure of antimalarials in these different epidemiological regions of malaria is a key prerequisite to resistance, endemic areas use the drugs most causing selection. In addition to, against all the drugs the least sensitive parasites were commonly observed in the epidemic region in the present study done in Kenya. This can be attributed to premunition, in low transmission sites most cases are symptomatic and selection occurs when antimalarial drugs are administered. Although, in higher transmission regions, symptomatic disease usually occurs when one is still young. Later on, malaria becomes less symptomatic which holds the infection in check at levels below the symptom threshold (Baird, Carlota, & Kevin, 2009).

The results of the current study between 2008 and 2019 period in the Kenyan population reported MQ exhibited increased parasite resistance over time in the 3 different time points while QN exhibited increased parasite sensitivity in the same period. Previous studies in Thai-Cambodian and Thai-Myanmar regions have reported increased prevalence of parasite resistance to MQ *in vitro* and *in vivo*, the results were monitored over four year observation period, on the other hand, QN sensitivity increased during the same period (Lim *et al*., 2009; Phompradit,

Muhamad, Wisedpanichkij, Chaijaroenkul, & Bangchang, 2014). The current study confirms similar results over a six year observation period in the Kenyan population, against MQ increased parasite resistance was reported, while QN sensitivity increased in the same period. The similar observations in both populations may be attributed to increase in *Pfmdr1* copy numbers which are associated with increased MQ resistance in the populations, treatment failure following AS-MQ combination therapy is also associated with increased copy number in SEA populations. *Pfmdr1* gene appears to be a significant component that plays a role in resistance to MQ. Morover, 1390I polymorphisms in *Pfmrp1* gene are associated with QN improved sensitivity in both populations.

The present study reported contradicting results of increased LU sensitivity and resistance between the 3 different time points over time during the study period between 2008 and 2019. A previous therapeutic efficacy study reported evidence of increased LU resistance in children in Zaire (Plucinski *et al*., 2015). However, Kamal and colleague was not in agreement with the conclusion of the study based on the study findings, they noted a number of limitations of the study, several of which were also acknowledged by the authors to conclude, no robust evidence of LU resistance in their reply letter on the previous article (Hamed & Kuhen, 2015). The present study reported contradicting results of increased LU sensitivity and resistance over time during the study period thus, proposes it is critical to monitor the potential development of resistance to current front-line antimalarials such as AL as the authors have attempted to do although careful surveillance of clinical efficacy, genetic changes in the parasite, and *in vitro* activity of artemether and LU remains an urgent priority, that was postulated by the previous study as well.

In the current study AQ was reported to have increased sensitivity over time during the study period that was between 2008- 2019 in Kenya. A previous study conducted in Kenya reported increased sensitivity to AQ over their study period which was between 2008-2011 (Eyase *et al*.,

2013). The current study conducted in the same population confirmed the findings as well, AQ was reported to have increased sensitivity over the study period that was between 2008- 2019, these observations could be due to decreased use of AQ in Kenya, causing a release of drug resistance pressure because AL had then been implemented as the primary treatment regime in the population.

5.3 Correlation of the polymorphisms in *Plasmodium falciparum* multidrug resistance protein 1 gene at codon 876 versus wild-type in antimalarial responses

Previous studies have shown the SNPs of *Pfmrp1* gene at codon 876 and 1390 as strongly associated with reduced susceptibility to MQ, ART, QN and LU (Dahlström *et al*., 2009; Gupta *et al*., 2014b; Veiga *et al*., 2011). However, the other two mutations at codons 191 and 437 have previously been associated with reduced sensitivity to CQ and QN (Dahlström *et al*., 2009; Gupta *et al*., 2014b). In the present study done in Kenya LU was reported to have reduced sensitivity in the 876V polymorphic parasites versus wild-type during the study period. Previous studies conducted in North-east and China-Myanmar border populations associated 876V mutations with reduced *in vitro* susceptibility to CQ in isolates from their populations and AL in African isolates. (Bai *et al*., 2018; Gupta *et al*., 2014b; Hao *et al*., 2013). The current study confirms similar results in the Kenyan population, LU was reported to have reduced sensitivity in the 876V polymorphic parasites, this could be attributed to I876V polymorphism in *Pfmrp1* gene found to be under significant selection pressure after AL treatment in an African population (Dahlström *et al*., 2009). Nonetheless, in the present study QN had different results with these previous studies, the same mutation was reported to be associated with reduced sensitivity to it, thus should be further investigated by future studies.

The present study conducted in a Kenyan population reported ART and CQ exhibited increased resistance at 876V polymorphic parasites versus wild-type during the study period. Previous

studies conducted on the Myanmar areas have reported a significance association of mutations in codon 876 of the *pfmrp1* gene confirmed slow parasite clearance time against ART and a 60.3% prevalence, was associated with increased CQ resistance in the same populations (Lo *et al*., 2016; Zhao *et al*., 2019). The present study conducted in Kenya corroborated similar results, increased resistance was observed against ART and CQ in 876V polymorphic parasites, this globally prevalent mutation could have been selected by extensive use of CQ in the past in both populations. The role of 876V mutations and its association with increased ART resistance observed in the the Kenyan population needs to be further investigated by future studies since ART resistance is popularly linked with P*fk13* mutations in the GMS.

In the present study, the correlation of polymorphic parasites versus wildtype among the potential mutations of *Pfmrp1* studied was only done at codon 876, since it had the highest frequency of mutations (58.9%) that was comparable among the others which were 191, 437 and 1390 that exhibited low frequencies of 3.1%, 3.3% and 7.1% respectively, which were impossible to correlate.

5.4 Limitations of the Study

Culture adapting samples from low transmission settings was challenging because most of them were unable to respond. Therefore, only a subset of 182 samples out of 300 available samples were analyzed after *in vitro* drug testing hence limiting the minimum sample size required for analysis. This limitation was minimized by culture adapting the samples with additional amount of serum to boost their growth. Furthermore, correlating other codons apart from 876 was impossible due to the low frequencies reported also with the missing set of *in vitro* data limiting it more. This can be minimized by testing samples using the *ex vivo* method to avoid culture adapting later. Additionally, the study was not able to compare trends per region since there were varying malaria burden across sites which impeded spatial analysis within Kenyan isolates.

CHAPTER SIX: SUMMARY OF FINDINGS, CONCLUSION AND RECOMMENDATIONS

6.1 SUMMARY OF FINDINGS

The objectives of this study were to determine the frequency of SNPs in *Pfmrp1* gene, *in vitro P.f.* drug susceptibility patterns and establish the correlation between the polymorphic in the gene and *in vitro* anti-malarial susceptibility profiles in Kenyan field isolates during implementation of ACTs. Results from the study showed polymorphisms at *Pfmrp1* codon I876V was the most frequent at 58.9% (162/275) mutants followed by F1390I, 7.1% (19/267) and S437A, 3.3% (9/274) while H191Y was the least at 3.1% (5/151). The *in vitro* sensitivity patterns of AQ and QN increased over time However, MQ exhibited increased resistance while LU exhibited contradicting results of increased resistance and sensitivity over time during the study period. Finally, infections with mutation at codon 876 were correlated with higher QN and LU 50% inhibition concentration during *in vitro* tests, suggesting reduced sensitivity and increased resistance against ART and CQ.

6.2 CONCLUSION

i. The frequency of *Pfmrp1* gene mutations in different geographical regions indicate selection pressure by multiple drugs on the *Pfmrp1* gene, which signifies its potential as an important candidate gene for monitoring drug resistance.

ii. MQ exhibited increased parasite resistance over time during the study period hence appeared to be undergoing positive selection. However, LU had contradicting results of increased resistance and sensitivity during the same period, in *vitro cross* resistance is expected to largely affect these 2 drugs because of their physicochemical similarities since they belong to the same class structurally.

iii. Kenyan study confirms previous African studies reports on the most frequent polymorphism 876V in correlation with *in vitro* anti-malarial resistance and is correlated with reduced susceptibility to LU.

6.3 RECOMMENDATIONS FROM CURRENT STUDY

According to the conclusions of this study, the following recommendations should be considered:

i. *Pfmrp1* gene mutations should also be a key marker in tracking resistance against ACTs in African populations and needs to be constantly monitored just as the other markers which are well documented.

ii. A positive correlation of *in vitro* cross-resistance between antimalarials in the aminoalcohols class such as LU and MQ popularly used in Kenya should be further investigated.

iii. The present study reported a correlation of QN with reduced *in vitro* susceptibility in 876V polymorphic parasites different with previous studies hence it needs to be further confirmed in more populations in Kenya.

6.4 RECOMMENDATIONS FOR FUTURE STUDIES

Based on the study findings, future studies should:

1. Urgently prioritize tracking selection of *Pfmrp1* gene mutations at codon 876 which appears to be prevalent in most African populations, it raises concerns especially in reinfections since it selects parasites against AL.
2. Clarify underlying reasons for the potential *in vitro* cross-resistance between ART derivatives and aminoalcohols, a clearer understanding of the parallel responses among these drugs have not been clarified.
3. Further investigate the role of 876V mutations and its correlation with increased ART resistance in polymorphic parasites as observed in the Kenyan population and in other populations as well.

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APPENDICES

Appendix 1: Sample cryopreservation, culture maintenance, parasitaemia determination, preparation of culture reagents and reconstitution of antimalarial drugs protocol

Preparation of Plain medium

To prepare 1L (Litre), 5.94g (Grams) of HEPES, 2g of glucose and 10.4g of 1640 RPMI was added to the beaker then topped up with 1L of distilled water. Stirring to mix then filtration in the biosafety cabinet using 0.2µl pore size filter unit done followed by storage at 4^0c for 2 weeks.

Preparation of PBS (Phosphate buffered saline) (Working buffer)

To prepare stock solution of PBS, 36.38g sodium phosphate monobasic and 59.29g of sodium phosphate dibasic was added to 1L beaker then topped up to 1L with distilled water. The contents were stirred to dissolve. 1X PBS was made by transferring 10ml of the stock solution into a cylinder then distilled water was added up to 1L and mixed.

Preparation of 10% giemsa stain

1 volume of giemsa was added to 9 volumes of working buffer 1x PBS then mixed thoroughly. It was used within 6 hours after preparation.

Preparation of complete medium with serum

Culture medium was prepared as described in the table below, a magnetic stirrer was used to dissolve all components in distilled water and subsequently filtered using 0.2µl pore size filter unit then stored at 4^0C for up to two weeks.

Volumes of reagents for 1L of culture medium

Reagent	Stock solution	Volume/weight
RPMI 1640+HEPES+ Glucose		42.4 ml
Hypoxanthine	1.45Mm	1ml
Sodium bicarbonate	75%	1.6ml
Heat inactivated ABO serum		5ml and 10ml for 10% and 20% respectively

Preparation of Lysis buffer containing SYBR green

To 700ml of distilled water, 15.76g of TRIS HCL (hydrochloric acid) was added and stirred to dissolve. The solution pH was adjusted to 7.5 using concentrated HCL. 160mg (milligrams) saponin and 16.0ml triton X-100 was added respectively. The volume was adjusted to 1L, the solution was mixed thoroughly to dissolve the contents and then filtration using 0.2µl pore filter unit to remove particulate matter was done followed by storage at room temperature for up to 6 months. Thawed one 30µl SYBR Green 1 was added to 15ml of Lysis buffer then mixed through pipetting in the dark, this solution was adequate for one 96 well plate.

Preparation of Red blood cells

Red blood cells are used in dilutions of culture to lower parasitemia and maintain hematocrit. RPMI medium was warmed to 37° C in water-bath or heater block. The red blood cells were then centrifuged at 500 g for 5 minutes . v/v RPMI medium was added to erythrocytes pellet to return to the original volume. It was then stirred 3–5minutes at room temperature on a shaker and centrifuged at 500g for 5 minutes. The supernatant was and the pellet of packed red cells washed two more times as above.

Antimalarial Drugs tested

SYBR Green I IC_{50} based *in-vitro* drug sensitivity assay was used to test *P. falciparum* field isolates, sensitivity against six antimalarial drugs procured as Chloroquine (CQ), Mefloquine (MQ), Quinine (QN), Artemisinin (ART), Amodiaquine (AQ) and Lumefantrine (LU). All the drugs were acquired from WWARN and they were in salt form.

Drug reconstitution for SYBR Green I IC_{50s} based sensitivity assay

Stock drug solutions at 1mg/ml was prepared by dissolving 5mg of drug in 5ml of 70% ethanol for QN and MQ or 100% DMSO for ART and LU. For CQ, 1.5 ml of deionized water plus 3.5ml absolute ethanol was used. Moreover, concentrated drugs (1mg/ml) was diluted further to desired starting concentrations in 10% CMS (complete medium serum), followed by 2-fold serial dilutions to generate 12 concentrations for IC_{50s} evaluation on 96 well plates to make mother plates. Starting concentrations (ng/ml) from highest to lowest was; CQ (2000-3.906), MQ (500-0.976), QN (4000-7.812), ART (200-0.381), AQ (200-0.381), The drug plates were stored at -80^{o}c up to 1 month or 12.5µl from each well of the respective drugs was transferred to new plates to form daughter plates for immediate drug testing (pre-dosed plates).

Cryopreservation of *P.falciparum* parasites procedure

The volume of the sample pellet was approximated, thrice the volume of glycerolyte was added to the pellet and mixed thoroughly. It was then transferred to a cryovial and not filled completely and later stored at a -80^{o}c freezer overnight before being transferred to liquid nitrogen for long storage.

Preparion of Malaria slides for microscopy

Thin and thick smears were prepared and let to air dry for a few minutes, afterwards only the thin smear was fixed in absolute methanol to prevent lysis. 10% giemsa stain was then prepared by mixing 1 part of giemsa to 9 parts of 1x PBS or distilled water, mixed thoroughly then poured on the slides and let to stain for 30 minutes prior microscopy under a 100x objective lens.

Culture maintenance during culture adaptation and growth monitoring

Cultures were maintained at 6% hematocrit for approximately 7-30 days accompanied by changing media, 20% complete media suspension and gassing in a gas mixture of 5% CO_2, 5% O_2 and 90% N_2 after every 48 hours. Parasitemia was checked through microscopy 3 times weekly until it reaches the recommended parasitemia for testing which was 0.3% to 1% if it was high it was adjusted by lowering it using washed RBCs. Parasitemia was estimated as a percentage of infected RBCs divided by total RBCs times 100%.

Appendix 2: Research Approval

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel: (254) (020) 2722541, 2713349, 0722-205901, 0733-400003, Fax: (254) (020) 2720030
E-mail: director@kemri.org, info@kemri.org, Website. www.kemri.org

KEMRI/RES/7/3/1 April 25, 2018

TO: DR. BEN ANDAGALU,
PRINCIPAL INVESTIGATOR

THROUGH: THE DIRECTOR, CCR,
NAIROBI

02/05/2018

Dear Sir,

RE: SERU PROTOCOL NO. 3628 *(RESUBMISSION OF INITIAL SUBMISSION):* EPIDEMIOLOGY OF MALARIA AND DRUG SENSITIVITY PATTERNS IN KENYA

Reference is made to your letter dated March 28, 2018 The KEMRI Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised application on April 6, 2018.

The SERU Secretariat acknowledges receipt of the following documents:

a. WR2454_MDR_protocol_v1.7 dated 12th March 2018, clean and tracked
b. WR2454_MDR_AdultParent_consent v1.5 MoH_12Mar2018 clean and tracked
c. WR2454_MDR_AdultParent_consent_KDF_v 1.0 dated_12Mar2018 clean
d. Removal letter of Maj Jacob Johnson
e. Appendix 1_attempts to find Maj Jacob Johnson
f. Local trabslations of the ICFs, Luo, Kiswahili, Kisii, Kipsigis
g. Certificates of translations for the ICFs.

This is to inform you that the Committee notes that the issues raised during the 272nd Committee B meeting of the SERU held on **February 21, 2018** have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day, **April 25, 2018** for a period of one year. Please note that authorization to conduct this study will automatically expire on **April 24, 2019.** If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **March 13, 2019**.

You are required to submit any proposed changes to this study to the SERU for review and the changes should not be initiated until written approval from the SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of the SERU and you should advise the SERU when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,

FOR: THE HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

In Search of Better Health

Appendix 3: Research Approval

MCMR-UWZ-C 4 January 2018

MEMORANDUM FOR Director, Human Subjects Protection Branch (HSPB), Walter Reed Army Institute of Research (WRAIR), 503 Robert Grant Ave., Silver Spring, MD 20910-7500

SUBJECT: Approval of the Minimal Risk Human Subjects Research Protocol **WRAIR #2454**

1. I approve the protocol, **WRAIR #2454**, entitled "Epidemiology of Malaria and Drug Sensitivity Patterns in Kenya," (Protocol Version 1.6, dated 21 December 2017), submitted by Ben Andagalu, MD, MSc, Deputy Chief, Malaria Drug Resistance Laboratory, Kenya Medical Research Institute (KEMRI)/Walter Reed Project (WRP).

2. This study is being funded by the U.S. Department of Defense Global Emerging Infections Surveillance and Response System (GEIS), Armed Forces Health Surveillance.

3. This protocol will replace the currently approved protocol WRAIR# 1384, entitled "Epidemiology of Malaria and Drug Sensitivity Patterns in Kenya." This is a combined clinical-laboratory based study to determine *Plasmodium* parasites drug resistance patterns and parasite genetic characteristics over time. The general objectives of this study are as follows: (1) To establish the *ex vivo/in vitro* drug resistance patterns of malaria parasites collected at various study sites in Kenya and to correlate these outcomes with the historical data obtained by the MDR laboratory. Information will be provided to GEIS and the Kenya National Malaria Control Program (NMCP), which may correlate our findings with their *in vivo* results. (2) To correlate genome-wide polymorphisms with antimalarial drug sensitivity tests determined by *in vivo* and *in vitro* methods respectively.

Up to 100 subjects per week will be enrolled from the surveillance sites located at the Kenya Ministry of Health facilities. Approximately 2500 subjects/year will be enrolled from the Kenya Defense Forces site. Subjects 6 months and older will be enrolled in this study.

4. The WRAIR Scientific Review Committee approved this protocol (Version 1.4, dated 14 August 2017) on 6 September 2017. There have been no scientific changes made between protocol Version 1.4, dated 14 August 2017 and Version 1.6, dated 21 December 2017.

5. The protocol (Version 1.5, dated 28 September 2017) was reviewed by the fully convened WRAIR Institutional Review Board (IRB) on 13 December 2017 and approved with stipulations. The protocol was determined to be a minimal risk study as per 32 CFR 219.111 and 45 CFR 46.111. This study also meets the criteria under 45 CFR 46.204, as it is research involving pregnant women, and 45 CFR 46.404, as it involves children participating on a not greater than minimal risk study.

The study team submitted the response and an updated protocol (Version 1.6, dated 21 December 2017) to the WRAIR IRB stipulations on 3 January 2018. The response to the stipulations was determined to be satisfactory on 4 January 2017.

6. The KEMRI Scientific and Ethics Review Unit (SERU) approval is still pending.

The Wellcome Trust Sanger Institute is not engaged in human subjects research as per the memorandum dated 9 October 2017.

The Medical Research and Materiel Command (MRMC) IRB approval/deferral is pending.

Appendix 4: Consent form

WRAIR 2454 KEMRI 3628: Epidemiology of malaria and drug sensitivity patterns in Kenya
Adult Consent Form (includes parental or legal guardian consent) for MoH sites

TITLE OF STUDY: Epidemiology of malaria and drug sensitivity patterns in Kenya
INSTITUTIONS: Kenya Medical Research Institute, Nairobi, Kenya; Walter Reed Project (United States Army Medical Research Directorate – Kenya), Nairobi, Kenya; Walter Reed Project, Kisumu, Kenya

PRINCIPAL INVESTIGATOR: Dr. Ben Andagalu, MD MSc

1 INTRODUCTION

You (your child) are being asked to participate in this research study. Participation in this study is voluntary. Refusal to participate will involve no penalty or loss of benefits to which you (your child) are otherwise entitled. You (your child) may discontinue participation at any time without penalty or loss of benefits.
This research study is supported by the United States Department of Defense. Funding for this study comes from the Global Emerging Infections Surveillance and Response System (GEIS), Armed Forces Health Surveillance Center. This study is a continuation of a study that has been running since 2007 that has helped describe how the malaria germs in different parts of Kenya respond to the drugs that are used to treat malaria

2 PURPOSE OF THE STUDY

You are being asked to participate in this study to learn about malaria germs and what drugs best treat the malaria found in this part of Kenya.

You (your child) will receive the malaria medicine prescribed by the hospital provider if it is necessary, and this testing will in no way affect your (your child's) treatment today.

3 WHO CAN PARTICIPATE IN THIS STUDY

1. Any person at least 6 months old can participate.
2. Expectant women 18 years and older can also participate in this study.
3. For civilian populations: If you (your child) are attending a Kenya Ministry of Health clinic and you (your child) live within a 25km radius of the study site and are aged 5 years and above, you (your child) can be included (if you are willing) in the subset of subjects who will have additional study visits.
4. For military populations: If you have joined the RTS and are currently attending the initial health assessments
5. You can participate in this study several times, as long as it is not in the same year. You will be asked to sign a new consent each time you are enrolled.

4 WHO CANNOT PARTICIPATE IN THIS STUDY

1. You (your child) are unwilling to give blood.
2. You are not capable of giving informed consent.
3. You (your child) are currently detained by the Kenya Government Department of Correctional Services.
4. You (your child) were previously enrolled in the study this year.
5. Your child who intends to get enrolled weighs less than 5 kg.

6. You (your child), in the opinion of the study doctor, would be affected by the drawing of blood (e.g., if you (your child) have low blood levels).

5 APPROXIMATE NUMBER OF VOLUNTEERS TAKING PART IN THIS STUDY

Up to 100 total volunteers per week, from all of the Ministry of Health sites where we are enrolling from, as well as approximately 2500 annually from the Kenya Defence Forces site.

6 PROCEDURES TO BE FOLLOWED FOR VOLUNTEERS RECRUITED FROM MINISTRY OF HEALTH SITES

If you (your child) agree to participate in this study, the procedures involve answering questions, having a brief physical examination, and donating blood. We will also ask you (your child) to visit us again on day 7 so we can see if the treatment was successful. Some volunteers who are willing will be asked to return additional times, on days 2, 3, 14, 28 and 42.

Blood samples will be taken in 2 ways. For the larger volume blood sample, which will be 5 ml (1 teaspoon), will be drawn from a vein in your (your child's) arm. For the smaller volume, 2-3 drops, a "finger stick" will be done.

If you agree, your (your child's) sample will also be tested for blood borne infections such as HIV, syphilis, West Nile Virus, Hepatitis B, Hepatitis C and Human T-Cell Lymphotropic Virus. This testing is optional and will not affect your participation in other study activities. The purpose of the testing is to select participants whose samples may be used for a special type of research called Controlled Human Malaria Infection (CHMI). Samples obtained from participants who test negative will be suitable for CHMI. Participants who test negative may be contacted later for confirmatory testing if needed. Those that test positive will still be used for malaria testing as explained above, and will be referred to the hospital clinician for further management of the blood borne infection.

Today's procedures: after signed informed consent, you (your child) will be asked some questions about your (your child's) age, occupation, village, residence, history of your sickness, symptoms, and antimalarial drug use. Then, you (your child) will undergo a brief procedure to provide a blood sample from the arm vein (5 ml or about 1 teaspoon) to test your (your child's) malaria germs in the laboratory and see what drugs will effectively treat your (your child's) malaria. The questions and blood drawing will take about 1 hour.

Day 7 procedures: you (your child) will be asked to return to this site 7 days from now, to answer questions only about whether the medication given improved the malaria illness. You (your child) will then be asked to donate 5 ml blood sample of blood from the arm vein for malaria testing. You (your child) will see the hospital clinician for further treatment. This visit will take approximately 1 hour.

Days 2, 3, 14, 28 and 42 procedures: if you (your child) agree to return for the additional study visits, at each visit, you (your child) will be asked questions only about whether the medication given improved the malaria illness. At each visit, you will be asked to donate 1-2 drops of blood by finger-stick for a malaria test. If the malaria test is positive, you will then be asked to donate a 5 ml blood sample from the arm vein for more laboratory testing. If the test is negative, you will not be asked for any more blood. Each return visit will take approximately 1 hour.
"Finger-stick" samples will be obtained from your (your child's) fingertip using a lancet device that minimizes pain, and makes only a small line cut (about 2-3 millimeter in length) on the finger tip skin. The finger tip is then squeezed, allowing a few drops of blood to be obtained for the laboratory tests.

All blood samples, except those used to diagnose your malaria by rapid diagnostic test, will be sent to a research laboratory in Kisumu, Kenya to test which drugs can kill your malaria germs. This is an experimental procedure, and due to the fact that the test takes a long time to run, the results of this test will not be immediately available to guide your treatment. The results of the rapid malaria test will be made known to you, while those conducted for the purposes of research will not be shared with you.

7 POTENTIAL RISKS AND DISCOMFORTS

The risk from participation in this study is small. There is some inconvenience associated with one or more clinic visits. There is the possibility of mild discomfort, bruising and very rarely infection at the arm or fingerstick site where the blood is obtained. There is also the possibility of feeling dizzy or fainting during or after a blood draw. The technician will use care to cause as little pain as possible and minimize the chance of infection after the blood draw. If the site should become infected, we will treat you (your child) with medication.

8 ANTICIPATED BENEFITS TO VOLUNTEERS

If you (your child) agree to be in the study, on the return visit(s), you (your child) will be tested for malaria to determine response to treatment. The study team will tell you the results. The test will show if the medication you (your child) received worked. If it did not, you (your child) will see the hospital clinician for an effective alternate anti-malaria medicine.

An indirect benefit to you (your child) is knowing that you (your child) have helped with a scientific study that may benefit other persons who become infected with malaria in the future by allowing us to test what drugs work best for treatment.

9 ALTERNATIVE TO PARTICIPATION

The alternative to participation in this research is to not participate. You (Your child) will still receive treatment for malaria and for any other illness you may have from the hospital provider even if you do not participate in this study.

10 PAYMENT FOR PARTICIPATION

There is no charge to participate in this study. In accordance with Kenyan custom, there is no direct compensation to volunteers for their participation.

For volunteers recruited from the MoH sites, support for public transportation to return to the study site for each follow up visit, and return home, will be provided to you (your child). The amount will be 500 Kenyan shillings per visit. Support for public transportation will also be provided for unplanned visits that are directly related to participation in this study.

Volunteers from the KDF sites are not expected to use public transport, and as such there will not be any direct compensation for transportation.

Other than medical care that may be provided and any other payment specifically stated in the consent form, there is no other compensation available for your participation in this research.

11 USE OF YOUR (YOUR CHILD'S) BLOOD SAMPLES

Your (your child's) blood samples will be stored and used only for the tests associated with this study. However, you (your child) may grant permission for the malaria germs in your blood to be used for other studies in the future, some of which may be done outside the country. You (your child) will be given a separate form to fill out which will allow you (your child) to say whether you (your child) will or will not allow your (your child's) blood samples to be used for future studies. The stored malaria germs for future studies will not have any items that could identify you as the original source, such as your name. No genetic testing will ever be done. There is no possibility that your samples could be used for developing a commercial product.

12 DURATION OF PARTICIPATION

Today, you (your child) will answer questions and donate blood today. This will take about 1 hour.

For volunteers from the MoH sites, on day 7, you (your child) will answer some questions and donate blood. If you agree to be in the group that returns up to 6 times, you will answer questions and give a blood sample on days 2, 3, 7, 14, 28 and 42 days after treatment. Each visit will last approximately 1 hour. For participants who accept to be tested for blood borne infections, there may be an additional contact to perform confirmatory testing. There will be no further clinic visits needed from either group.

13 ASSURANCE OF CONFIDENTIALITY

Records relating to your (your child's) participation in the study will remain confidential to the extent possible. Research records will be kept in a locked file at Walter Reed Project, Kisumu, Kenya. Your (your child's) name will not be used in any report resulting from this study. All computerized records and laboratory specimens will contain

only a unique study number for you (your child), not your (your child's) name, or any other personal identifying information. Computer records will be password protected and accessed by authorized study personnel only. Research records will be kept until all data analyses are completed.

14 PARTICIPATION AND WITHDRAWAL

Your (your child's) participation in this study is voluntary. You (your child) have the right to leave this study at any time. Refusal to participate or study discontinuation will not result in a penalty, a compromise of your medical care, or a loss of benefits to which you (your child) are otherwise entitled. For volunteers at KDF sites, refusal to participate or study discontinuation will not affect your training.

In the event that you (your child) exit the study before its completion, regardless of the reason, we encourage you to participate in the scheduled blood sampling for follow up malaria testing on Day 7 if you were found to have malaria.

15 WITHDRAWAL OF PARTICIPATION BY THE INVESTIGATOR

The investigator may withdraw you (your child) from participating in this research if circumstances arise which warrant doing so. If you (your child) become ill during the research, beyond what would be expected from a malaria infection, you (your child) may have to drop out, even if you (your child) would like to continue. The investigator will make the decision and let you (your child) know if it is not possible to continue. The decisions may be made either to protect your (your child's) health and safety, or because it is part of the research plan that volunteers who develop certain conditions may not continue to participate.

You (your child) may also be removed from this study without consent if:
a) you (your child) do not follow the study procedures
b) in the opinion of the study physicians, it is in your (your child's) best interest,

16 NEW FINDINGS

You (your child) will be informed of all malaria test results (and the test results for the blood borne infections if you agreed to testing), as they relate to your current treatment. Results obtained in the research laboratory testing will not be made available to you, but will be made available to the Kenyan Ministry of Health and the KDF (for KDF volunteers).

During the study, you (your child) will, however, be informed of any significant new findings (good or bad) such as changes in the risks or benefits resulting from participation in the research or new alternatives to participation, which might cause you (your child) to change your (your child's) mind about continuing in this study. If new information is provided to you (your child), consent to participate in this study will be re-obtained

17 REVIEW OF RESEARCH RECORDS

It should be noted that representatives of the US Army Medical Research and Materiel Command and KEMRI are eligible to review research records as a part of their responsibility to protect human subjects in research. The research records will be made available only to investigators and clinical hospital personnel who may need this information to treat you (your child), or to members of the Ministry of Health who require this information for administrative reasons.

18 PERSONS TO CONTACT FOR ANSWERS TO RESEARCH RELATED QUESTIONS

If you think you (your child) have a medical problem related to this study, you may report this to Principal Investigator of the Study - Dr. Ben Andagalu, Malaria Drug Resistance Laboratory, United States Army Medical Research Directorate/KEMRI (The Walter Reed Project), Kisumu, Kenya, Tel: 0716004851 or +254202023858.

19 PERSONS AND PLACES FOR ANSWERS REGARDING YOUR RIGHTS AS A RESEARCH SUBJECT

If during the course of this study, you have questions concerning the nature of the research, you should contact the Principal Investigator of the Study - Dr. Ben Andagalu, at the Walter Reed Project, telephone +254716004851 or +254202023858. If you are not satisfied, you may also contact the Secretary of the Scientific and Ethics Review Unit, c/o Kenya Medical Research Institute, P.O. Box 54840, Nairobi, Kenya, tel. 020-272-251 or 0722205901. The Scientific and Ethics Review Unit of Kenya Medical Research Institute is a body that is independent of the study team.

20 RIGHTS OF RESEARCH SUBJECTS

You (your child) may withdraw consent at any time and discontinue participation without penalty. You (your child) are not waiving any legal claims or rights because of your participation in this research study. If you have questions regarding your rights as a research subject, you may contact: The Director, Regulatory Affairs Office, Walter Reed Project, P.O. Box 54, Kisumu, Kenya or telephone +254202023858 and/or the Secretary of the Scientific and Ethics Review Unit, c/o Kenya Medical Research Institute, P.O. Box 54840, Nairobi, Kenya, tel. 020-272-251 or 0722205901.

CONSENT: By signing this form, you agree that you have read the information provided above, or that it has been explained to you. You have talked to a member of the study team about the study. You have also been given an opportunity to ask questions and these have been answered to your satisfaction. You agree that we have talked to you about the risks and benefits of the study, and about other choices. You (your child) may drop out of the study at any time, and nothing will change about your (your child's) medical care. A copy of this form will be given to you (your child).

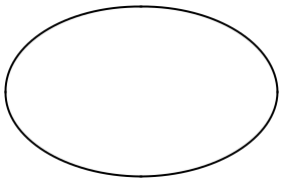
If Subject is a minor Name of Subject: _____

Printed name of participant/parent/guardian: _____

Signature of participant/parent/guardian: _____

Date: _____

Thumbprint of adult subject/parent/ guardian if unable to sign



WITNESS: I have witnessed the explanation of the research study to the participant/parent/guardian. The participant was given an opportunity

to ask questions, and the participant's questions, if any, were answered.

Printed name of witness: _____

Signature of witness: _____

Date: _____

INDIVIDUAL OBTAINING CONSENT: I certify that I have explained to the above participant/parent/guardian the nature and purpose of this study, potential benefits, and possible risks associated with participation in this study. I have answered any questions that have been raised.

Printed name of individual obtaining consent: _____

Title: _____

Signature:_____Date: _____

INVESTIGATOR

Printed name: _____

Signature:_____ Date: _____

WRAIR 2454 KEMRI 3628: Epidemiology of malaria and drug sensitivity patterns in Kenya

Consent for Testing for Blood Borne Infections, Future Research Use and Long-Term Blood Sample Storage

Adult Consent Form (includes parental or legal guardian consent)

TITLE OF STUDY: Epidemiology of malaria and drug sensitivity patterns in Kenya

INSTITUTIONS: Kenya Medical Research Institute, Nairobi, Kenya; Walter Reed Project (United States Army Medical Research Directorate – Kenya), Nairobi, Kenya; Walter Reed Project, Kisumu, Kenya

PRINCIPAL INVESTIGATOR: Dr. Ben Andagalu,

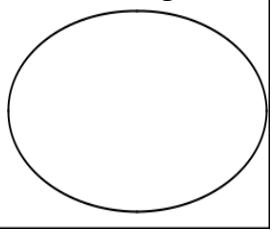
You (your child) agree that the investigators may store your (your child's) blood samples that contain malaria germs indefinitely for possible use in other research studies. No human genetic studies will be undertaken with these samples. Your (your child's) decision to allow storage of blood samples is optional. Your (your child's) samples, if stored, may be shipped to laboratories located outside the country for further analyses. If you agree, your (your child's) samples will also be tested for blood borne pathogens as explained in the main consent form.

CONSENT FOR STORAGE OF BLOOD SAMPLES: By signing this form, you **AGREE** that you have read the information provided above, or that it has been explained to you. You have also been given an opportunity to ask questions and these have been answered to your satisfaction. A copy of this form will be given to you (your child).
Sign **YES if you agree** and **NO** if **do not agree** to allow storage of blood samples for future research.

-_____ **YES**. I will allow my (my child's) samples to be stored for future use

-_____ **NO**. I do not want my (my child's) samples to be stored for future use

Thumbprint of adultsubject/parent/ guardian if unable to sign



If Subject is a minor Name of Subject: _____

Printed name of participant/parent/guardian: _____

Signature of participant/parent/guardian: _____

Date: _____

WITNESS: I have witnessed the explanation regarding long term storage of blood to the participant/parent/guardian. The participant was given an opportunity to ask questions, and the participant's questions, if any, were answered.

Printed name of witness: _____

Signature of witness:_____ Date:_____

Printed name of individual obtaining consent for long term storage:

_____

Title: _____

Signature:_____ Date: _____

CONSENT FOR TESTING FOR BLOOD BORNE INFECTIONS:

By signing this form, you **AGREE** that you have read the information provided above, or that it has been explained to you. You have also been given an opportunity to ask questions and these have been answered to your satisfaction. A copy of this form will be given to you (your child).

Sign **YES if you agree** and **NO** if **do not agree** to allow testing for blood borne infections.

-_____ **YES**. I will allow my (my child's) samples to be tested for blood borne infections

-_____ **NO**. I do not want my (my child's) samples to be tested for blood borne infections

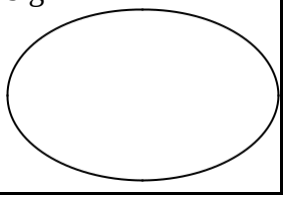
If Subject is a minor Name of Subject: _____

Printed name of participant/parent/guardian: _____

Signature of participant/parent/guardian: _____

Date: _____

Thumbprint of adult subject/parent/ guardian if unable to sign



WITNESS: I have witnessed the explanation regarding testing for blood borne infections to the participant/parent/guardian. The participant was given an opportunity to ask questions, and the participant's questions, if any, were answered.

Printed name of witness: _____

Signature of witness:_____ Date:_____

Printed name of individual obtaining consent for blood borne infections testing:

_____

Title: _____

Signature:_____ Date: _____

INVESTIGATOR

Printed name: _____

Signature:_____ Date: _____