MOLECULAR MONITORING OF PfATPase6 SINGLE NUCLEOTIDE POLYMORPHISM (SNPs) IN RELATION TO ARTEMISININ RESISTANCE IN WESTERN KENYA

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Molecular monitoring of PfATPase6 single nucleotide polymorphism (SNPs) in relation to artemisinin resistance in Western Kenya

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DECLARATION

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

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DEDICATION

This thesis is dedicated to my family.

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ABBREVIATIONS AND ACRONYMS

ACT	Aretmisinin Combinational Therapies
AL	Artemether-Lumefantrine
ANOVA	Analysis Of Variance
AFRIMS	Armed Forces Research Institute Of Medical Science
BECA	Biosciences Eastern and Central Africa
Ca^{2+}	Calcium Ions
C.I	Confidence Interval
CO_2	Carbon Dioxide
CMS	Complete Media With Serum
CQ	Chloroquine
CRPMI	Complete RPMI 1640
CV	Culture Volume
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
g	Gravitational acceleration
HRPII	Histidine Rich Protein II
IC_{50}	Half Maximal Inhibitory Concentration
ILRI	International Livestock Research Institute
JKUAT	Jomo Kenyatta University Of Agriculture And Technology
Log	Logarithm

Mdr1	Multi Drug Resistant Gene 1
Mg	Milligram
Ml	Milliliter
MSF	Malaria SYBR Green I-Based Fluorescence Assay
MUSCLE	Multiple Sequence Comparison By Log-Expectation
N_2	Nitrogen
$NaHCO_3$	Sodium Bicarbonate
ng/ml	Nanograms Per Milliliter
O_2	Oxygen
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
Pf	*Plasmodium Falciparum*
PfATPase6	*Plasmodium Falciparum* Adenosinetriphosphatase6
QA	Quality Assurance
QC	Quality Control
RBC	Red Blood Cells
RFU	Relative Fluorescence Units
RNA	Ribonucleic Acid
RPM	Revolution Per Minute
RPMI	Roswell Park Memorial Institute 1640 Series Of Cell Culture
1640	Medium
RT	Room Temperature (18 To 30 Degrees)

SERCA	Sarco/Endoplasmic Reticulum Ca2.-Atpase
SNP	Single Nucleotide Polymorphism
SP	Sulphadoxine-Pyrimethamine
WHO	World Health Organization
µs	Micro Second
µl	Micro Liter
µM	Micro Molar

ABSTRACT

P. falciparum resistance to drugs is a major public health problem. Early last decade, a concern was raised regarding resistance to Sulphadoxine-Pyrimethamine by falciparum malaria in the country. Following recognition that S-P therapy was failing, in April 2004, replacement options resulted in a decision to adopt artemether-lumefantrine (AL), as the first line therapy in Kenya. Subsequent to the policy change by the Kenyan government, it was reported elsewhere that artemisinin resistance is associated with the S769N mutation in the PfATPase6 gene. Furthermore, N569K and L89E mutations have been investigated in West Africa. The aim of this study was therefore to investigate whether mutations have occurred in the PfATPase6 genes of parasites circulating in Western Kenya and if so, whether the mutations have affected malaria treatment by artemisinin drugs.

One hundred and fifty *P. falciparum* field isolates were collected in 2009 from blood obtained from consenting patients at Kisumu, Kericho and Kisii district Hospitals. A nested PCR and sequencing assay was developed to analyze the genotype polymorphisms of the PfATPase6 involving S769N, N569K and L89E changes. Bioinformatics tools were applied for these analyses. The tools included DNA BASER for contig assembly of nucleotide sequences, NCBI ORF finder to translate the nucleotide sequences to protein code, MUSCLE 3.6 to align the genetic sequences and GENEDOC software to view multiple sequence files. *In-vitro* culture of the parasites was followed by artemisinin sensitivity testing of the parasites using Malaria SYBR Green I-based fluorescence assay.

Results were analyzed by Kruskol-wallis one-way ANOVA test using graph pad prism software.

Western Kenya PfATPase6 genes presented polymorphisms at five codons, *viz.*: 569, 679, 630, 639 and 657. Kisumu District hospital had the highest prevalence of 73.07% at codon S679F followed by Kericho with 70% and Kisii with 12.5%. At codon N569K, Kericho District Hospital had the highest prevalence of 37.5% followed by Kisumu District Hospital at 23% and Kisii District Hospital with 20%. Codons A630S and T657P had equal prevalence of 7.69% in Kisumu District Hospital and 12.5% at the Kisii District Hospital. G639D codon mutation was at 2.7% occurring only in Kericho District Hospital. All mutations observed were rare, except the PfATPase6 S679F in 61.36% and PfATPase6 N569K found in 25% of parasites. No polymorphisms were observed at codons 89 and 769. In general, the highest rate of polymorphism was observed in Kisumu District Hospital, suggesting sustained drug pressure at this site compared to the other sites studied. Amongst 92 samples tested and sensitive to artemisinin, 42.39% had S679F mutations while 17.39% had N569K. 15.21% had both S679F and N569K mutations. This might suggests a synergistic relationship between the two mutations. Only 2 (2.89%) parasite isolates had all five mutations present. Analysis of the IC_{50} values to determine association between the mutations and resistance to artemisinin revealed no such co-relation.

It can be concluded that in 2009, *P. falciparum* parasites circulating in Western Kenya were mainly polymorphic at codons 569 and 679 in the PfATPase6 genes. Continual

usage of ACT in the region may influence this polymorphism hence suggesting need for close and sustained monitoring of these polymorphisms in this gene as well as artemisinin sensitivity in western Kenya.

CHAPTER ONE

1.1 Introduction

Malaria is caused by the infection by intracellular parasites of genus *Plasmodium* transmitted by female anopheles mosquitoes. The most dangerous of this genus is the species *P. falciparum* (Mendis, *et al*. 2001). At present 100 countries are considered malariuos (WHO, 2006) with almost half of the population at risk being in the Sub-Saharan Africa. The malaria problem persists in spite of more than a century of efforts to eradicate or control it. Half of the world's population is at risk of malaria and an estimated 243 million cases led to nearly 863 000 deaths in 2008 (WHO, 2009 a).

Resistance to antimalarials is a major drawback in effective malaria control in Sub-Saharan Africa. *P. falciparum* has developed resistance to the cheap and safe chloroquine and to sulfadoxine-pyrimethamine (WHO, 2006). Therefore, artemisinin-based combination therapies are recommended for use in the whole of sub-Saharan Africa (WHO, 2012; Murakami, *et al*. 1990). Fifteen out of 43 sub-Saharan African countries have already adopted artesunate plus amodiaquine as first-line drug and the rest are at various stages of preparation for changes to the same regimen or to artemether plus lumefantrine. To date no relevant clinical resistance to artemisinins has been reported. However, the long-term usefulness of ACT in high transmission areas remains unclear (Kimura, *et al*. 1999). Therefore, as ACT is becoming widely used in sub-Saharan Africa, the need for regular and comprehensive surveillance of resistance has been recognized. This should include *in-vitro* and *in-vivo* drug efficacy assays,

pharmacokinetic analyses and the monitoring of molecular markers associated with resistance to the various components of ACT.

1.2 Statement of the problem

P. falciparum resistance to drugs remains a major public health issue in Western Kenya. In 2001, the year National Malaria Strategy was officially launched, concerns were raised about growing evidence of a decline in SP clinical efficacy as measured through the then standard WHO day 14 clinical and parasitological sensitivity test. By mid-June 2001 studies showed that its failure rates by day 14 were in excess of 25% (East African Network for monitoring Antimalarial Treatment, 2003).

Following recognition that SP was failing, replacement options resulted in a decision to adopt AL as the recommended first line therapy in Kenya (Amin A, *et al.* 2007). Recently, studies have shown a relationship between the SERCA PfATPase6 gene and artemisinin efficacy, and pointed it out as a potential molecular marker for resistance. The goal of this work was to describe the baseline polymorphism of PfATPase6 gene in Western Kenya.

1.3 Justification

Laboratory studies have shown that genetically stable and transmissible artemisinin-resistant rodent malaria parasite could be selected through prolonged exposure of drug-sensitive lines to low and increasing levels of artemisinins (Afonso, *et al*. 2006). The discovery of the PfATPase6 gene provides a new target for drug makers and for researchers on improving the killing power of artemisinins. This gene that encodes the

pump (PfATPase6) can be monitored in parasites to see if it mutates to make the parasite resistant to artemisinins. With this in mind it will be possible to look for and anticipate resistance, instead of responding when it happens. Various studies done before on the mutations on the PfATPase6 gene have indicated the need for continuous monitoring of the gene for mutations that may result in development of resistance to artemisinin combination therapy which is widely used in Africa for malaria treatment. This study is thus aimed at detection of any mutations of the gene in Kenya's most endemic regions and there after give the necessary recommendations to the ministry of health.

1.4 Research Question

Are there single nucleotide polymorphisms in the *P. falciparum*-derived PfATPase6 amongst plasmodium parasites circulating in Western Kenya and if any, what effect do the polymorphisms have on the sensitivity of the parasites to artemisinin?

1.5 Hypothesis

1.5.1 Null hypothesis

There are no mutations on the *P. falciparum* ATPase6 gene at locus S769N, N569K, and L89E that results in resistance of artemisinin-combined therapy in Western Kenya.

1.5.2 Alternative hypothesis

There are mutations on the *P. falciparum* ATPase6 gene at locus S769N, N569K, and L89E that results in resistance of artemisinin-combined therapy in Western Kenya.

1.6 Objectives

1.6.1 General objective

To determine whether *P. falciparum* parasites circulating in Western Kenya are or have developed resistance to artemisinin malarial therapy and its association with PfATPase6 gene.

1.6.2 Specific objectives

1. To assess the presence of previously characterized artemisinin-related SNP mutations in PfATPase6 in *P. falciparum* parasites circulating in Western Kenya.
2. Identify any new SNPs mutations in the PfATPase6 gene in isolates circulating in Western Kenya.
3. To determine and compare IC_{50} of *P. falciparum* parasites mutants and ascertain the association between the mutation(s) observed the IC_{50}.

CHAPTER TWO

2.1 Literature Review

It was reported that on the *P. yoelii*, a rodent malaria parasite, Ca^{2+}-ATPase gene actively participates in the fine-tuning of the cytoplasmic concentration of Ca^{2+} in the parasites (Murakami, *et al*. 1990). The importance of the regulation of Ca^{2+} for malaria parasites has been indicated by the fact that disturbance in Ca^{2+} transport by Ca^{2+} ionophores arrests the growth of *P. falciparum*, the human malaria parasite (McAlister and Mishra,1983). The PfATPase6 is an ATP-dependent ionic pump from the Plasmodium endoplasmic reticulum membrane involved in cation transport. Artemisinin and its derivatives negatively regulate this cationic pump (Kimura, *et al*. 1999; Dahlstrom, *et al*. 2008).

Recently it was hypothesized, and later proved, that artemisinin interact and selectively inhibit PfATPase6, the only SERCA-type Ca^{2+}-ATPase in the *P. falciparum* genome (Jambou, *et al*. 2005; Uhlemann, *et al*. 2005). A subsequent in vitro study in French Guyana showed that *P. falciparum* with elevated IC_{50} values for artemisinins shared a specific point mutation at codon S769N of the ATPase6 locus and this mutation has been proposed as a molecular marker of resistance against artemisinin (Jambou, *et al*. 2005).

Recently, a study was carried out in Tanzania to asses resistance of artemisinin in that region (Mugittu, *et al*. 2006). In this study, S769N changes in the PfATPase6 were not reported even though the study sites included Mkuranga, a coastal region close to urban Dar es Salaam with a high rate of uncontrolled use of artemisinin monotherapy (Falade, *et al*. 2005). The findings concluded that resistance to artemisinin had not yet

been selected for in Tanzania and are consistent with the high (~94%) artemether plus lumefantrine parasitological cure rates recorded in a multi-country efficacy trial including Tanzania's coastal region (Mugittu, *et al*. 2006). In 2006–2007 periods a study was carried out in China to investigate whether the S769N mutation had occurred in field isolates. The results of six samples could not be obtained due to failure of PCR amplification, but no S769N mutation was detected in any of the 95 samples successfully amplified (Zhang, *et al*. 2008). The results indicate that the S769N mutation in the PfATPase6 gene had not occurred in China, suggesting that artemisinin resistance had not yet developed. This does not necessarily imply that such mutations would never occur in China, hence close monitoring of this mutation was recommended.

Three new point mutations on the PfATPase6 protein were discovered in Niger but only one, N569K, had a high prevalence of 17.2% of the population (Ibrahim, *et al*. 2009). The conclusion of this study was that the PfATPase6 gene is polymorphic at the 569 codon. As ACT is getting more and more used, other polymorphisms in the PfATPase6 gene in association with phenotypic *in-vivo* and/or *in-vitro* drug efficacy tests require monitoring. Indeed, Happi *et al,* 2008 studied resistance of malaria parasite to artemisinin therapy and found that codon L89E of the PfATPase6 is also polymorphic. They assessed *P. falciparum* mdr1 (Pfmdr1) gene polymorphisms and copy numbers as well as PfATPase6 gene polymorphisms in 90 Nigerian children presenting with uncomplicated falciparum malaria and enrolled in a study of the efficacy of AL. All pre- and post treatment samples as well as gametocytes harbored a single copy of the Pfmdr1 gene and the wild-type allele (L89) at codon 89 of the PfATPase6 gene. Considering all evidence

available to them, the authors suggested that polymorphisms in the Pfmdr1 gene are under AL selection pressure and that Pfmdr1 polymorphisms may result in reduction in the therapeutic efficacy of this newly adopted combination treatment for uncomplicated falciparum malaria in Sub-Saharan countries of Africa (Happi, *et al*. 2008).

More recently, reports of high failure rates associated with ACTs, as well as *in vitro* drug-susceptibility data, suggest the possibility of clinical artemisinin resistance along the Thai–Cambodian border (Vijaykadga, *et al*. 2006; Jambou, *et al*. 2005; Noedl, *et al*. 2008). Results from these studies indicated that some patients had parasite-clearance times that were prolonged and the plasma drug concentrations after the first dose were classified as adequate (Vijaykadga, *et al*. 2006). For these subjects, the IC_{50} for dihydroartemisinin were up to 4 times the geometric mean for cured patients and almost 10 times that for the reference clone, accession number GU797316. Widespread resistance would not only be a setback for Cambodia, which has managed to substantially reduce its malaria caseload over the past decade (from 175 000 cases in 1997 to 62 000 cases in 2008), but also countries in sub-Saharan Africa could see their gains in combating child mortality curtailed. “Chloroquine was lost over a period of 20 years” and “child mortality in Africa increased because of an increase in malaria”, If these parasites develop full-blown resistance to artemisinin derivatives, it could be a global health disaster, warn experts (WHO, 2009 b).

Monitoring of drug resistance in field isolates and antimalarial activities of new compounds has been made possible by use of *in-vitro* drug susceptibility testing of malaria parasites. The MSF assay used in this study has been validated under various

culture conditions (Bacon, *et al*. 2007). In this assay, reference strains of *P. falciparum* were cultured *in-vitro* by using standard conditions in complete medium with and without phenol red before they were dispensed into 96-well plates pre-dosed with chloroquine, mefloquine, or quinine. The IC_{50} results of this assay are comparable to those obtained by the HRPII assay with fresh clinical samples without removal of white blood cells. The advantage of this assay is that parasites are assessed in response to drugs without interference from host factors, such as acquired immunity and pharmacokinetic profiles (Noedl, *et al*. 2005). It can also be used with cultivated parasites to assess new antimalarials (Noedl, *et al*. 2001; Wongsrichanalai, *et al*. 2001; Noedl, *et al*. 2002) or can be used with fresh clinical isolates to conduct epidemiological studies (Russell, *et al*. 2003; Noedl, *et al*. 2005).

An inherent problem associated with the use of cultured parasites for *in vitro* testing is the potential for the selection of a dominant clone during the culturing phase, which could alter the IC_{50} values (Russell, *et al*. 2003). The use of fresh isolates overcomes this laboratory-induced artifact and gives true values for the parasite populations obtained from infected patients (Matthias, *et al*. 2010). In general, the MSF assay is used routinely to conduct surveillance for drug resistance in *P. falciparum* with fresh or cultured parasites because it is quick and cost-effective in the determination of IC_{50} values (Basco 2003 a, Basco 2003 b, Bacon, *et al*. 2007).

The principle of the MSF assay is that it detects the quantity of malaria DNA in infected erythrocytes as a measure of parasite growth and propagation. An increase in parasite-specific DNA is an indication of growth of the parasites while parasite growth

inhibition due to antimalarial drugs is indicated by stagnation or a drop in the parasite-specific DNA. Continued validation on MSF techniques have been conducted where the capability of this assay was expanded by including antibiotics and antifolates in the drug panel and testing folic acid-free growth conditions and also evaluated a more expansive panel of antimalarials in combination with various drug assay culture conditions commonly used in drug sensitivity screening for their activity against *P. falciparum* strains D6 and W2 (Jacob D. *et al*, 2007).

CHAPTER THREE

3.1 Materials and Methods

Some of the chemicals used in this study were supplied by Sigma-Aldrich, Spruce street St. Louis, which included; 0.5M EDTA pH 8.0, Ethidium bromide, Magnesium chloride, Glucose, Hepes, Agarose, 4μg/ml hypoxanthine, 20mM Tris (pH 7.5), 5mM EDTA, 0.008% Triton X-100 and 10X Tris-borate-EDTA buffer. RPMI 1640, powdered medium (10.4 grams) was obtained from Invitrogen, Incorporation and water for cell culture applications was from Lonza, USA. Deoxyribose nucleotide triphosphates was obtained from Roche Molecular Systems USA, 100% DMSO was supplied by Arkema Incorporation, while absolute ethanol (200 proof) and Giemsa Modified Azure Blend, EM were obtained from Fisher Scientific. Methanol was bought from Fisher Scientific. Sodium bicarbonate, 3.2% (vol/vol) of catalog number SH40003-11 was supplied by Thermo Fisher Scientific Incorporation Waltham. Proteinase K stock solution-(>600 mill-Arbitrary units /ml, QiAmp DNA mini kit and QIAmp spin column tube were supplied by QIAGEN Incorporation Turnberry Lane. All these company are located in the United States of America. The Yamato Sterilizer SM510, model no. 21670 was from Yamato scientific Company located in Japan.

Artemisinin drug (100ng/ml) and two *P. falciparum* control strains, the Sierra Leone "D6" chloroquine-sensitive and the Indochina "W2" chloroquine-resistant were donated by Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington DC. The strains were maintained aseptically as previously described by Trager and Jensen, 1976 to attain replication robustness prior to assay. *P.*

falciparum field isolates were obtained from three West Kenya field sites in accordance with WRAIR 1384, KEMRI 1330 protocol.

Other consumables used in the study included 3mm filter paper from Whatman Maidstonein the United Kingdom. Alcohol Swabs from Fisher healthcare, Osborn Street, Keyport, U- Taq polymerase got from Roche Molecular Systems, Incorporation, zipper plastic bags from Bradley bags Firestone, and diamond grip gloves from Micro flex corporation. Needles and syringes were obtained from BD, Becton Drive Franklin Lakes, , Eppendorf reference Pipettes from Eppendorf Brinkmann Instruments Incorporation, Aerosol Resistant Tips from Molecular Bioproducts Incorporation. Safe-lock Tube 2.0ml, natural lot number Y135876J were bought from Eppendorf Incorporation. All these companies are also located in the United States of America. The 96 well micro-culture plates were obtained from Nunc Incorporation roskildle in Denmark. Fast reaction Tube caps and ABI prism big dye terminator v3.1 cycle sequencing kit were supplie by Applied Biosystems

The following primers were supplied by Applied Biosystems, USA: F1- GGA AGA GGT TAT TAA GAA TGC , R1-GCT TCA ACA TTT CCT TCA TC, F2-TAT TAG ATA TGA AAC ATA AAA ATC and R2-GGA GTT TTA TTA CCA ACA CTC AAT TCA were used in the nested PCR of codon S769N , P17.1-TGG ATC AAT AAT ACC TAA TCC ACC TA, P17.1rev AAT ATT GTT ATT CAG AAT ATG ATT ATA A , P17-AGC AAA TAT TTT CTG TAA CGA TAA TA and P17 rev- TGT TCT AAT TTA TAA TAA CAT CT GT were used in the nested PCR for codons 569, 630, 639, 657 and 679 while PfATP6-1OF – CAA ATA AGA AGG ATA AA TCA CCA , PfATP6-1OR- TCA

TAA ATA CAC GTA TAC CAG CC ,PfATP6-1Nest-F AAA ATA AAT ACC ACA TCA ACA CAT and PfATP6-1Nest-R TCA ATA ATA CCT AAT CCA CCT AAA were used in nested PCR of codon 89.

Equipments used were also from different suppliers including; Gene Amp PCR system 9700 by Applied Biosystems, 454 Sequencing System by Roche Diagnostics Corporation, Hague Road Indianapolis while the Lab-line refrigerator and freezer were from General Electric Appliances Park. Thermo scientific CO_2 incubator, model number RC0500D-9-VBC was bought from Asheville, Electrophoresis tank from Nasco Company. Tecan Genios Plus multi-well plate reader was purchased from MTX Lab Systems, Incoporation, Beckman coulter Biomek 2000 from Beckman Coulter, Incoporation. Sartorius weighing balance; model no. CP64 was obtained from Precision Weighing Balances, Isotemp 215 water bath from Fisher Scientific Fairlawn, StreilGARD2 Advance class 2 Biological safety cabinet model number. SG624NT was from the Baker Company Drawer Stanford while Graph pad prism software used in the analysis was obtained from The Graph pad prism inc. in San Diego all located in the United States of America.

Eppendorf 5804R centrifuge was from Eppendorf AG Barkhausenweg in Hamburg Germany, Microwave oven from LG electronic in Korea while the Biosafety cabinet Lab Caire PCR work station was from Lab Caire systems Clevedon United Kingdom and the Ultra-low temperature freezer by Sanyo Electronic Company in Japan.

3.2 Study sites

The three district hospitals are located in areas that are endemic to malaria. They were ideal site for sample collection because most patients with malaria symptoms usually visit the district hospitals for check up. The Walter Reed Project/Kemri also has its staff located in these hospitals thus making it easy for sample collection. Three hospitals in Western Kenya *viz*: Kisumu district hospital, Kericho district hospital and Kisii district hospital

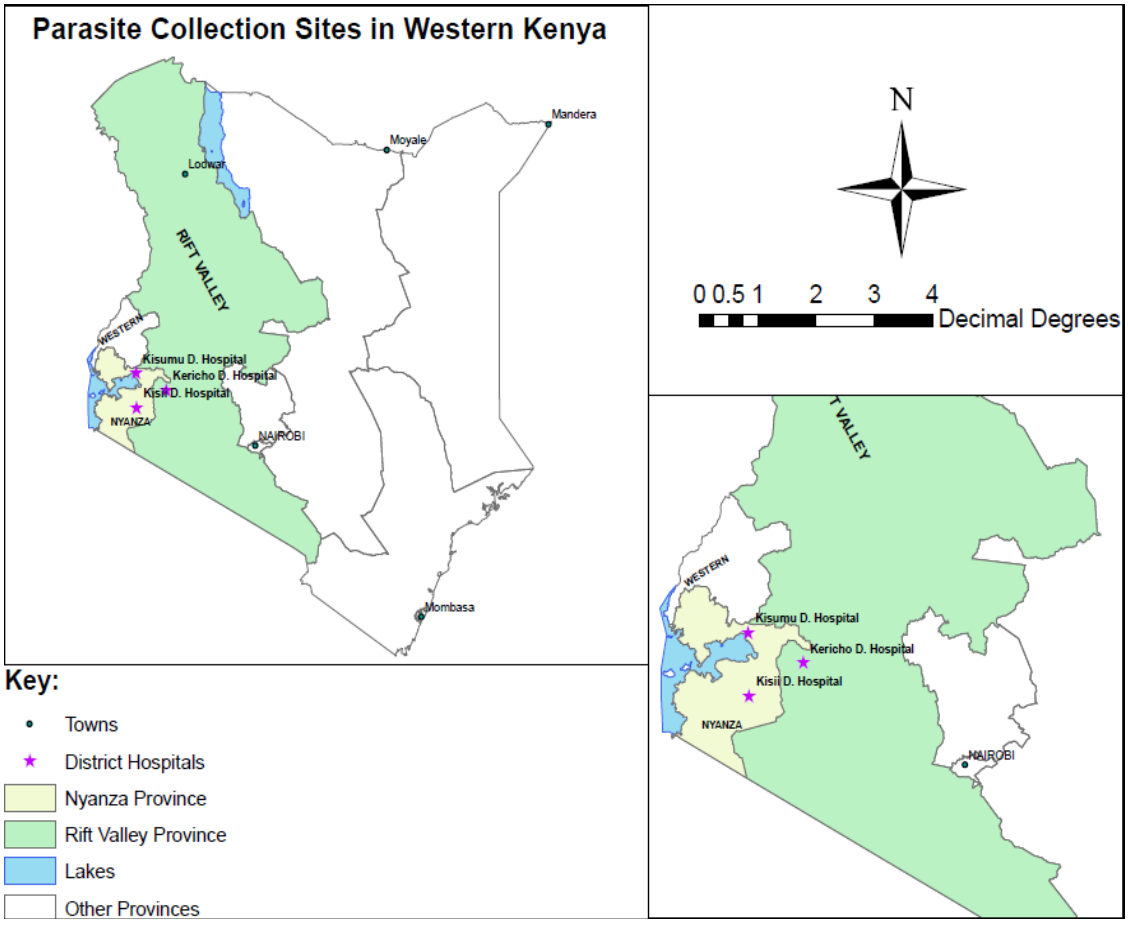

Figure 1: Map showing western Kenya and the three district hospitals where the study was conducted. The Kisumu, Kericho and Kisii district hospitals were the sites involved in recruiting participants in the study.

3.3 Study populations

The study population consisted of individuals six months or older presenting with uncomplicated malaria infection as determined by thick/thin smear blood microscopy. One hundred and fifty consenting randomly selected individuals living in proximity to the health facilities were included in the study. Uncomplicated malaria patients were used because patients showing signs of complicated malaria are most likely to be anemic because of loss of red blood cells through lysis by parasites and are usually treated using the second line malaria drugs such as quinine and not ACTs, which were used in this study.

Sample size was calculated using the Raosoft® sample size calculator formula (Raosoft, 2004). The software has been used before by the U.S. Army Research Institute for the Behavioral and Social Sciences in a study to determine occupational analysis program in the U.S. Army. (E. Brady, 2004)

$$x = Z(^{c}/_{100})^{2} r\,(100-r)$$

$$E = \text{Sqrt}\,[^{(N-n)\,x}/_{n\,(N-1)}] = 5.06\%$$

Confidence level at 90% = 1.645

$$n = ^{N\,x}/_{((N-1)\,E2\,+\,x)} = 150$$

Where N is the population size, r is the fraction of responses that you are interested in, and $Z(c/100)$ is the critical value for the confidence level c, E is the margin of error, which is the amount of error that you can tolerate.

This calculation is based on the Normal distribution, and assumes availability more than 30 samples.

From the previous years' statistics, the average number of new malaria cases (N) analyzed at the Malaria Drug Resistance testing laboratory was 345 patients. On average, Kisumu district hospital had 204 patients (59%), Kisii district hospital had 63 patients (18%), and Kericho district hospital had 78 patients (23%). Using these statistics, each collection site was assigned sample numbers according to the average percentage number of samples collected the previous years. Therefore, Kisumu district hospital was assigned 90 samples, Kericho district hospital 33 samples and 27 samples from Kisii district hospital.

3.4 Inclusion criteria for participants

Consenting individuals presenting with a malaria parasite positive thick/thin blood smear were included in the study. Furthermore, these individuals must have had uncomplicated malaria (not anemic), be six months or older, must not have taken any antimalarial drugs two weeks prior to introduction into the study and must have been resident in the region.

3.5 Exclusion criteria of participants

Individuals who had a negative thick/thin blood smear test for malaria parasites were excluded. Those who did not consent for inclusion in the study were also excluded from the study. Infants younger than six months, pregnant women, persons with severe malaria, and persons who have had malaria treatment two weeks prior to presentation to the study were not eligible.

3.6 Sampling procedures

From patient who met the inclusion criteria, approximately 20 µl of finger- prick blood was spotted on a piece of 3 MM filter paper and air-dried. The dried filter paper samples were stored in individual zipper plastic bags with dryer at -20degrees celsius awaiting DNA extraction. Venous whole blood (2ml) was also collected from the cephalic vein on the arm using a syringe and needle and stored in EDTA (1.8mg/ml) to prevent clotting. This sample was used for in *in-vitro* culture and drug sensitivity testing.

3.7 Laboratory procedures

3.7.1 DNA extraction

The extraction of DNA from the dried blood spots was performed following the QIAamp® DNA Mini Kit protocol for extraction of dried blood spots. Briefly water baths set at 85 degree Celsius, 56 degree Celsius, and 70 degree Celsius were used at various stages of the process. All centrifugation steps were carried out at room temperature. A total of three punched-out circles from a dried blood spot were placed into a 1.5ml micro centrifuge tube followed by addition of 180µl of buffer ATL and incubated at 85 degrees Celsius for 10 minutes. This was followed by a brief centrifugation (6000xg for 30 seconds) in a bench top centrifuge to collect any drops of the buffer on the micro centrifuge tube lid.

Proteinase K stock solution (20µl of) was added and briefly mixed. This was followed by an incubation of the mixture at 56 degree Celsius for 1 hour. Then a brief

centrifugation (6000xg for 30 seconds) was carried out. After centrifugation, 200µl of buffer AL was added to the sample and mixed thoroughly by vortexing then incubated at 70 degree Celsius for 10 minutes before briefly centrifuging again at 6000xg for 30 seconds in a bench top micro-centrifuge to remove drops from inside the lid. Absolute ethanol (200µl) was added to the sample and mixed thoroughly by vortexing followed by another brief centrifuging at 6000xg for 30 seconds.

The mixture was then applied carefully to the QIAamp spin column (in a 2ml collection tube) without wetting the rim, the lid closed and centrifuged at 6000xg for 1 minute. The QIAamp spin column was then placed in a clean 2ml collection tube and the tube containing the filtrate was discarded. The QIAamp spin column tube was carefully opened and 500µl of buffer AW1 was added without wetting the rim followed by closing of the cap before centrifugation at 6000xg for 1 minute. The QIAamp spin column was again put in a clean 2ml collection tube and the collection tube containing the filtrate was discarded.

The QIAamp spin column was then carefully opened and 500µl of buffer AW2 was added without wetting the rim and centrifuged at full speed (20,000xg) for 3 minute. In order to eliminate carryover of buffer AW2, the QIAamp spin column was placed in a new 2ml collection tube followed by discard of the collection tube with the filtrate. The tube was then centrifuged at 20,000xg for 1 minute.

The QIAamp spin column was finally placed in a clean 1.5ml micro-centrifuge tube and the collection tube containing the filtrate discarded. The QIAamp spin column

was carefully opened before adding 150µl of buffer AE or distilled water, followed by an incubation step at room temperature for 1 minute, and then centrifuged at 6000xg for 1 minute to obtain purified DNA.

3.7.2 Nested PCR for the various SNPs

The fragments of PfATPase6 gene were amplified by nested PCR using locus-specific primers shown below:

S769N

For the primary cycle, the primers used in this assay were F1 and R1. Primers F2 and R2 were used for the second (nested) cycle. The cycling conditions included denaturation at 94 degrees Celsius for 40 seconds followed by primer annealing at 46 degrees Celsius for 1 minute and a strand extension at 72 degrees Celsius for 1 minute. This was repeated for 35 cycles followed by a final strand extension step at 72 degrees Celsius for 10 minute. The amplification products were incubated at 4 degrees Celsius for both primary and secondary cycles.

N569K

Primers P17.1 and P17.1rev were used in the primary amplification. Primer P17 and Primer P17 rev were used for the nested amplification. The cycling conditions included denaturizing at 94 degrees Celsius for 40 seconds, primer annealing at 46 degrees Celsius for 1 minute and strand extension at 72 degrees Celsius for 1 minute. This was repeated for 35 cycles followed by a final strand extension at 72 degrees Celsius for 10 minute.

L89E

Primers PfATP10F and PfATO10R were used in the primary amplification. Primers PfATPase6-Nest-F and PfATP6-Nest-R were used in the nested amplification step. The cycling conditions included denaturation at 94 degrees Celsius for 1 minute, primer annealing at 45 degrees Celsius for 1 minute and a strand extension at 72 degrees Celsius for 1 minute. These were repeated for 40 cycles with a final extension step at 72 degrees Celsius for 10 minute.

In all PCR reaction assay set ups, the 50 µl PCR mixtures contained primers at 0.25 µmol/L final concentration, 2 mmol/L $MgCl_2$, 240 µmol/ L of each deoxyribonucleotide triphosphate, 2 U *Taq* polymerase and template DNA (1 µl). The primary amplicons were diluted in distilled water in a ratio of 1:9, and then 3 µl of the diluted amplicons were introduced into a 50 µl nested amplification mixture. The nested PCR products were resolved by electrophoresis on 2% agarose gels, stained with a 0.5 µg/ml ethidium bromide solution and visualized under ultra-violet light.

3.7.3 PCR product clean-up

A 3x reaction volume of PB buffer was added to the PCR products and agitated briefly to mix the contents. A PCR clean-up column (Qiagen®) was placed into a 2ml micro centrifuge tube and the extracted product added to the PCR column and centrifuged at 12000xg for 1 minute. The filtrate was discarded then the PCR column was returned to the 2ml micro centrifuge tube. After W2 buffer (700ul) was added onto the column, it was

centrifuged at 12,000xg for 1 minute and filtrate discarded. The PCR column was again returned into the 2ml micro centrifuge tube and 400ul of W2 buffer was added into the column and centrifuged at 12,000xg for 1 minute. The PCR column was finally transferred into a clean 1.5 micro centrifuge tube to elute the DNA by adding 25-30ul of elution buffer (pre-warmed at 65 degrees Celsius) to the centre of the membrane and left to stand for 1 minute at room temperature. These were then centrifuged at 12,000xg for 1 minute to collect the clean PCR products.

3.7.4 Nucleotide sequencing

Sequencing reactions were carried out using ABI PRISM Big Dye terminator v3.1 Cycle Sequencing kit as specified by the manufacturer's protocol. Briefly the DNA templates to be used for sequencing were free of both organic residues (that is phenol and chloroform) and high salts (that is, DNA precipitated using ethanol and sodium acetate needs to be thoroughly rinsed with 70% ethanol before resuspension). The DNA sequencing reaction included a final reaction volume of 10 µL. This was made up of 2 µL of water, 3 µL of 5mM $MgCl_2$, 1 µL of primer, 3 µL of template and 1 µL of ABI Big Dye version 3.1. The amount of DNA used was 15ng for a 1kb PCR product. Sequencing conditions included a 96 degrees Celsius denaturation stage for a period of 30 seconds, an annealing temperature at 50 degrees Celsius for 15 seconds and elongation at 60 degrees Celsius for 4 minutes. This was repeated for 30 cycles to obtain the final product. The final product (amplicons) were purified and sent for sequencing at Beca-ILRI Nairobi.

3.8 Parasite cultures and *in- vitro* drug susceptibility assays

3.8.1 Preparation of RPMI 1640 basic media

RPMI 1640 basic media consisted of RPMI 1640 powder combined with 2 grams of glucose and 5.95 grams HEPES dissolved to homogeneity in 1 liter of de-ionized water and sterilized with a 0.2μM filter.

3.8.2 Preparation of complete RPMI 1640 media

Complete RPMI 1640 media, used for all parasite culture drug dilutions and drug testing, consisted of RPMI 1640 basic media with 10% (volume/volume) human ABO pooled plasma, 3.2% (volume/volume) sodium bicarbonate and 4μg/ml hypoxanthine added. Complete RPMI 1640 media was stored at 4 degrees Celsius and used within 2 weeks.

3.8.3 Preparation of drug solutions

All these procedures are sensitive to contamination and required use of personal protective equipment. Wearing of gloves and flushing them with the 70% ethanol was therefore mandatory at all the stages of this procedure. First a 3" X 3" weighing paper was weighed and the weight was tarred before adding the drug up to 5mg. The drug was then transferred into sterile 15ml polystyrene centrifuge tubes. The tubes were labeled with the name of the drug, concentration in mg/ml and date prepared. The drug was then dissolved in 5ml 100% DMSO which gave a stock concentration of 1mg/ml.

3.8.4 Preparation of mother drug template

From the stock, the starting concentration for artemisinin 100 g/ml was prepared using the complete RPMI 1640 culture medium as diluents. 350 µL of the artemisinin starting concentration was added to Column 1 manually followed by at two fold serial dilution in complete culture medium spanning from well 1 to well 12 using the automated laboratory workstation biomek 2000 to make the mother plate. 12.5 µL from each of the 96 wells of the plate was transferred on to corresponding wells of the working plate. The mother plate was used within thirty days.

3.8.5 Preparation of cultures and hematocrit controls for drug screen

The SYBR green I-based *in-vitro* drug susceptibility assay technique described in details earlier (Bacon, *et al*. 2007; Jacob, *et al*. 2007) was used with modifications. Briefly, *P. falciparum* reference isolates in clones "chloroquine resistant" W2, "chloroquine sensitive" D6 and chilled field isolates obtained from distant sites were first cultured at 6% hematocrit, to reach 3% to 8% parasitemia, for 7 to 30 days, to establish parasite replication robustness. Prior to drug testing, the parasite culture was adjusted to 2% hematocrit and 1% parasitemia, and cultured in the presence of antimalarial drug aliquots in complete RPMI 1640. For field isolates from nearby sites, parasitemia ≥1.5 was lowered to 1% or hematocrit alone lowered for ≤ 1% parasitemia.

For drug assay, the ten varying dilutions serial two fold dilutions (10 concentrations and the first row was control wells) of the drugs (12.5 µl/well) were

dispensed into 96-well micro culture plates by a semi automated micro dilution technique using Beckman 2000 laboratory work station. A total of 100 µl of cell medium mixture at 2% hematocrit and parasites at 1% was added to each well, and the plates were incubated for 72 hours in a gas mixture (5% CO_2, 5% O_2, and 90% N_2) at 37 degrees celsius. The plates were subsequently freeze-thawed for hemolysis. For further haemolysis, 100µl of lysis buffer containing SYBR green I (1x final concentration) were added directly to the plates and gently mixed by using the Beckman Coulter Biomek 2000 automated laboratory work station.

3.8.6 Plate reader set up

The plates were incubated for 5 to 15 minutes at room temperature in the dark. Parasite growth inhibition was quantified by measuring the per well relative fluorescence units (RFU) of SYBR green 1 dye using the Tecan Genios Plus® with excitation and emission wavelengths of 485 nm and 535 nm, respectively, and with the gain set at 60.

3.8.7 Determination of IC_{50}

Data in form of relative fluorescence units (RFUs) was outputted directly to MS Excel. For analysis, the readouts were aligned with corresponding drug doses and analysis performed by Graph-pad Prism software. The drug concentrations (x value) were transformed using X=Log[X] and plotted against the counts (y values) and the data was analyzed by non-linear regression (sigmoid dose-response/variable slope equation) to yield the IC_{50}. (This is a 5 parameter analysis that includes IC_{50} value, top of curve, and bottom of curve, Hill slope, and R2). Results in form of IC_{50} per sample were entered on

an excel spreadsheet. Data was pulled per sample, per codon and compared with IC_{50} for cloned (chloroquine-resistant W2 and chloroquine-sensitive D6) to describe susceptibility status. The statistical method used was Kruskal-Wallis H test for non-parametric comparisons, using the graph pad prism software. Dunn's multiple comparison test was also used to analyze correlation between the various mutations. [Graphpad® software, Inc]

3.9 Quality control of laboratory procedures

Great care was taken to keep the sample preparation, PCR master-mix preparation and agarose gel areas of the laboratory well separated to prevent cross-contamination. Multiple negative controls in every PCR run also included to control for possible contamination. All results were discarded if any negative control was positive. To control for inhibition products in starting materials, each PCR reaction was performed in duplicate. Quality control was maintained by analysis of both negative and positive quality control samples alongside the test samples to check for any contaminations or if the masters mix reagents are working optimally. In the culture and susceptibility assay all procedures (except centrifugation) were performed in a Class II, type B2 Biological Safety Cabinet. The bio-safety cabinet surface was wiped down with 70% ethanol using paper towels at the beginning and the end of every day.

3.10 Data management

3.10.1 Data storage: Laboratory results form

The laboratory results-form included the following information: sample identification number, sample collection site, nested PCR results and visualization of amplicons and sequencing results of PCR products. The data was recorded in laboratory books kept under lock and key and in a computer that was password protected.

3.10.2 Nucleotide Sequence analysis

DNA sequence data derived from the amplicons were sent back and bioinformatic approaches were applied to analyze the sequence data. These bioinformatic tools included, DNA BASER software to determine contig sequences of the forward and reverse DNA sequences. After obtaining the contig, the ORF Finder (Open Reading Frame Finder) was used to translate nucleotide sequences to protein code. The published PfATPase6 amino acid code (Accession number EF564343) was used in comparing with the Western Kenya field isolates sequences. The multiple sequence comparison by log- expectation (Edgar R.C 2004) was used to align the amino acid sequences of field isolates and the published reference strain. GENE DOC software was used to view the multiple sequence alignment data. The nucleotide sequences obtained were deposited into the GeneBank database website to obtain a GenBank Accession Numbers for ease of identification (Appendix 3).

3.10.3 Ethical Considerations

An informed consent was obtained from the patients before any specimens were collected (Appendix 1). Confidentiality was achieved by use of laboratory reference numbers instead of the patients' names. Therapy for malaria was administered to patients participating in the study and a follow up done to check on their recovery progress. This study was part of a larger ongoing study title; "Epidemiology of Malaria and Drug Sensitivity Patterns in Western Kenya (v.6 dated 25th August 2008.)" The study protocol was reviewed and approved for implementation by the KEMRI Scientific Steering Committee during its 150TH SSC meeting held on 7th October 2008, SSC # 1330 (Amendment), (Appendix 4). It was also reviewed and approved by the KEMRI/National Ethical Review Committee at the 160th meeting held on 4th November 2008. Documentations for these approvals are attached (Appendix 5).

CHAPTER FOUR

Results

4.1 Genetic Polymorphism in the PfATPase6 genes

Genetic polymorphisms were observed at positions 1707 (Tyrosine to Adenine), 1888 (Guanine to Tyrosine), 1916 (Guanine to Adenine), 1969 (Adenine to Cytosine) and 2036 (Cytosine to Tyrosine) [3D7 numbering system] representing codons 569, 630, 639, 657 and 679 respectively in the PfATPase6 gene from parasites obtained from patients in Western Kenya. Codon S679F of PfATPase6 gene had the highest mutation incidence of 73.07% recorded at Kisumu District hospital followed by Kericho District Hospital with 70%. In contrast, only 12.5% of specimens collected from the Kisii District Hospital during the same period had this S679F mutation. Patients' blood specimens from Kericho District Hospital had the highest percentage of mutation at codon N569K in 37.5% of samples. This was followed by Kisumu District Hospital with mutations in 23% of the samples and the least percentage of mutation was observed in samples from the Kisii District Hospital, with only with 20% mutation. Codons A630S and T657P had an equal occurrence of 7.69% mutations at Kisumu District Hospital. Likewise, similar mutations occurred at Kisii District Hospital with a frequency of 12.5%. Codon mutation at position 639 was observed only in samples obtained from Kericho District Hospital at 2.7%, which was very rare (Table 1).

Table 1: Percentage results of PfATPase6 mutation. The table contains results of the percentage mutations as observed at the three district hospitals.

	S769N		S679F		N569K	
	% PER D. HOSP.	TOTAL %	% PER D. HOSP.	TOTAL %	% PER D. HOSP.	TOTAL %
KDH	0		73.07		23	
KCH	0		70		20	
KSI	0	0	12.5	61.36	37.5	25

	L89E		A630S		T657P		G639P	
	% PER D. HOSP.	TOTAL %	% PER D. HOSP.	TOTAL %	% PER D. HOSP.	TOTAL %	% PER D. HOSP.	TOTAL %
KDH	0		7.69		7.69		0	
KCH	0		0		0		10	
KSI	0	0	12.5	6.81	12.5	6.81	0	2.7

4.1.1 PfATPase6 L89E mutations

Overall, all (100%) of samples obtained from the patients at enrollment were successfully analyzed for the presence of the mutation at codon 89 of the PfATPase6 gene. The mutant PfATPase6 (E89) allele was not detected in the samples. These samples did not require nucleotide-sequencing process since the principle used was based on a PCR followed by RFLP approaches. The restriction sites were engineered in the primers in order to differentiate between mutant and wild-type sequences. Visualization by gel electrophoresis showed no evidence of amplified DNA (Table 1, Figure 2).

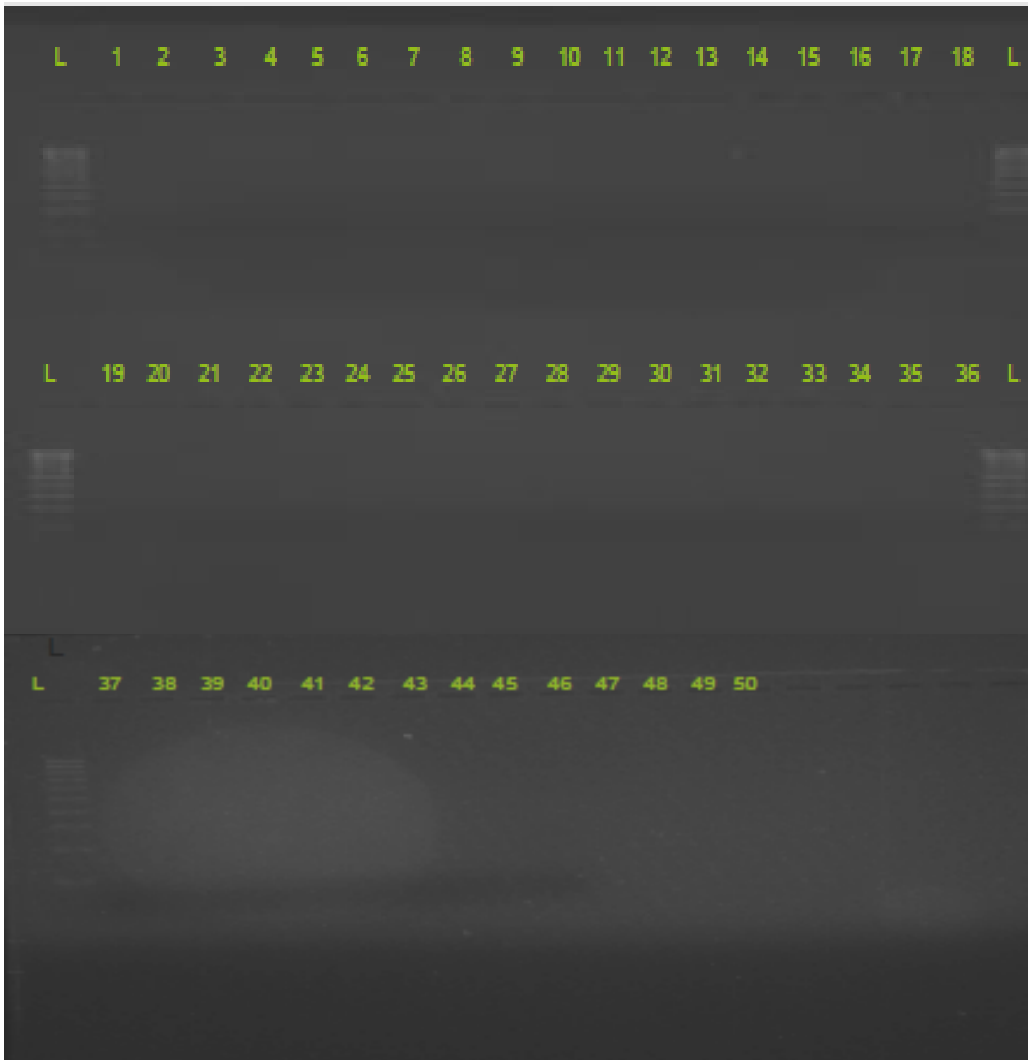

Figure 2: Gel photo for codon L89E showing no bands.

4.1.2 PfATPase6 S769N mutation

A 437-base pair fragment in the PfATPase6 S769N gene was amplified by nested PCR in field samples. Gel electrophoresis of the secondary PCR products indicated that they were of the expected sizes (Table 1, Figure 3).

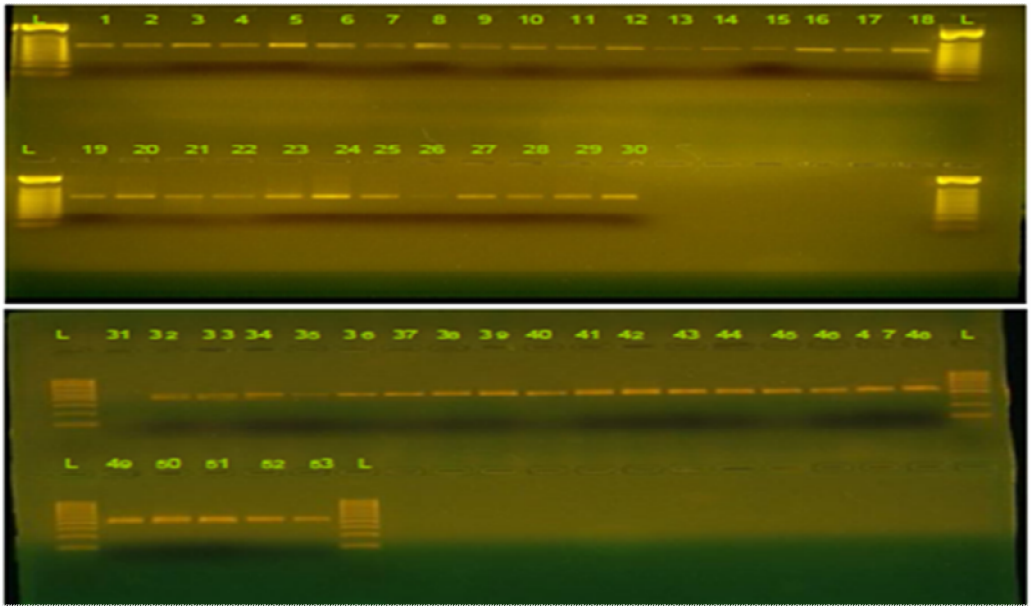

Figure 3: Gel photo for codon S769N showing bands of 437 base pairs**.**

Sequencing assays were successfully performed for the nested PCR products of the rest of the isolates. Bioinformatics tools were then employed, which included multiple sequence comparison by log-expectation (MUSCLE) to align the DNA sequences then used Gene Doc software to visualize the alignment it was observed that there were no mutations to codon S769N on the PfATPase6 gene (Table 1). Figure 5 shows the graphical representation of the analysis. The amino acid sequence of the parasite isolate at this codon is shown in Figure 7a.

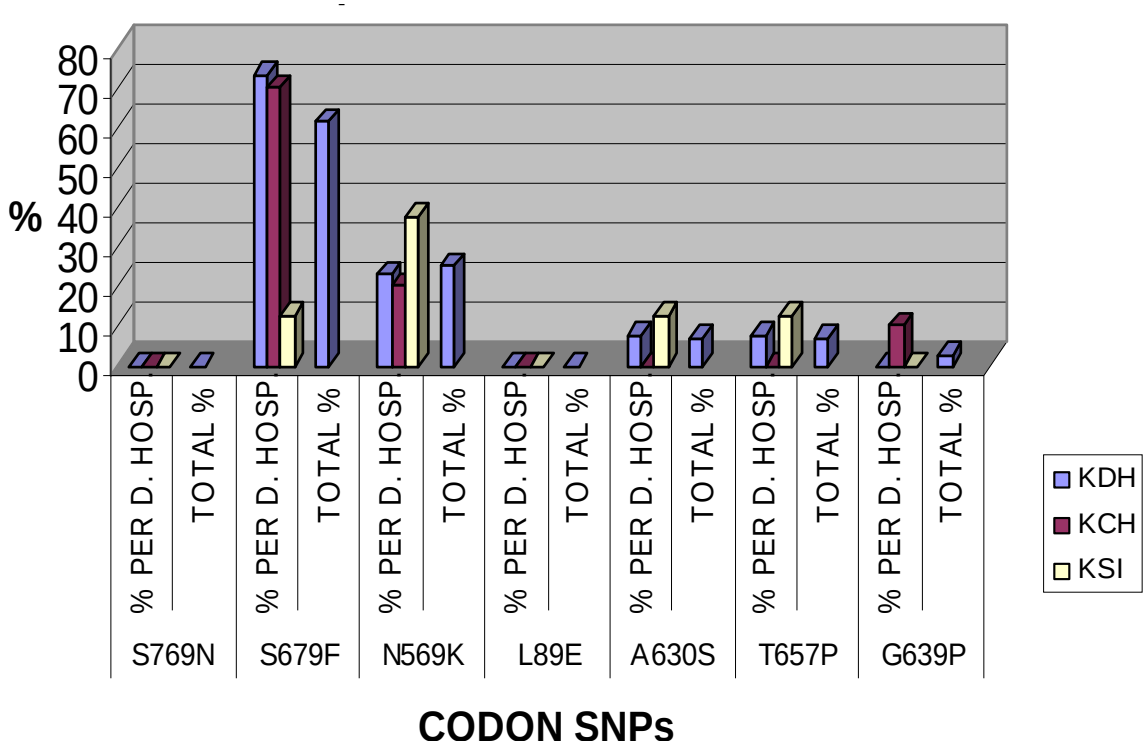

Figure 5: PfATPAse6 mutations graphical results per district hospital

4.1.3 The PfATPase6 N569K mutation

A 798-base pair fragment in the PfATPase6 N569K gene was amplified by nested PCR in field samples. Gel electrophoresis of the secondary PCR products indicated that they were of the expected sizes. The PfATPase6 N569K mutation was observed in 25 % of the samples (Table 1, Figure 4).

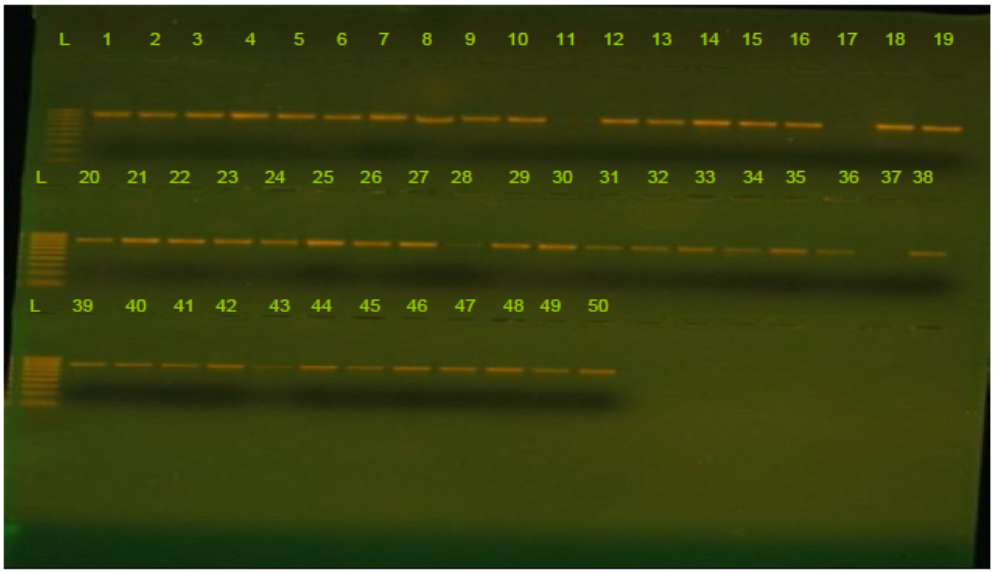

Figure 4: Gel photo for codon N569K, A630S, G639D, T657P showing bands of 798 base pairs**.**

Figure 5 shows the graphical representation of the analysis. The amino acid sequence of the parasite isolate at this codon is shown in Figure 7b.

4.1.4 Additional PfATPase6 mutations

Some other mutations observed in the PfATPase6 field isolates from Western Kenya in other loci include mutations at codon A630S (6.81%) and G639D (2.7%). New and novel SNPs observed in Western Kenya include S679F (61.36%) and T657P (6.81%). Of these, S679F is the most significant in the region (Table 1). These mutations were observed in loci that were around codon 569 (798 bases) and thus they are represented in figure 4. Figure 7c shows the amino acid sequences of these loci.

4.2 *In -vitro* culture and sensitivity assay

A total of 92 samples were successfully analyzed through *in-vitro* culture and sensitivity assay to determine their IC_{50} values. These were compared to the IC_{50} values of the wild type isolates in order to determine whether there is significant differences in sensitivity of parasites with these mutation and those that did not have the mutations. The medians of the IC_{50} values among the mutants did not vary significantly that is, $p>0.05$ at a confidence level of 95%. Amongst 92 samples tested and sensitive to artemisinin, 42.39% had S679F mutations while 17.39% had N569K. 15.21% had both S679F and N569K mutations. Only 2.89% parasite isolates had all five mutations present. Analysis of the IC_{50} values to determine association between the mutations and resistance to artemisinin revealed no such co-relation (Figure 6, Appendix 2).

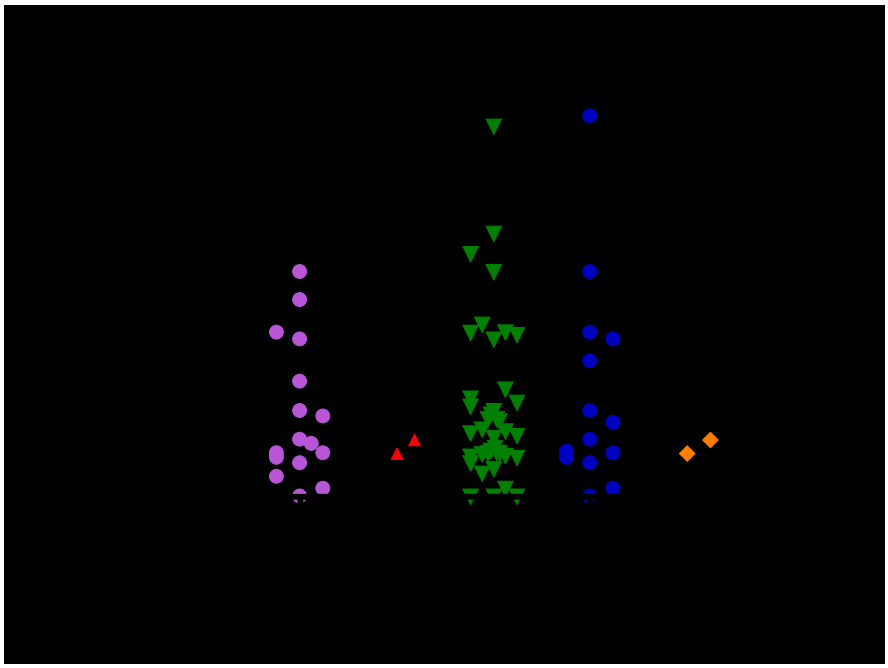

Figure 6: IC_{50} graph plot showing relationship between mutation and IC_{50}.

Analysis of the IC_{50} values to determine co-relation between the mutations revealed that the p-value greater than 0.05 at 95% confidence interval. Further analysis by Dunn's multiple comparison test of the various mutations amongst each revealed that there was no correlation. That is, in comparing the Wild Type verses N569K, Wild Type verses T657P, Wild Type verses S679F, Wild Type verses N569K&S679F, Wild Type verses samples with all the mutation present, N569K verses T657P, N569K verses S679F, N569K verses N569K&S679F, N569K verses samples with all the mutation present, T657P verses S679F, T657P verses N569K&S679F, T657P verses samples with all the mutation present, S679F verses N569K&S679F, S679F verses samples with all the mutation present, N569K&S679F verses samples with all the mutation present all had p-values greater than 0.05 (Appendix 2).

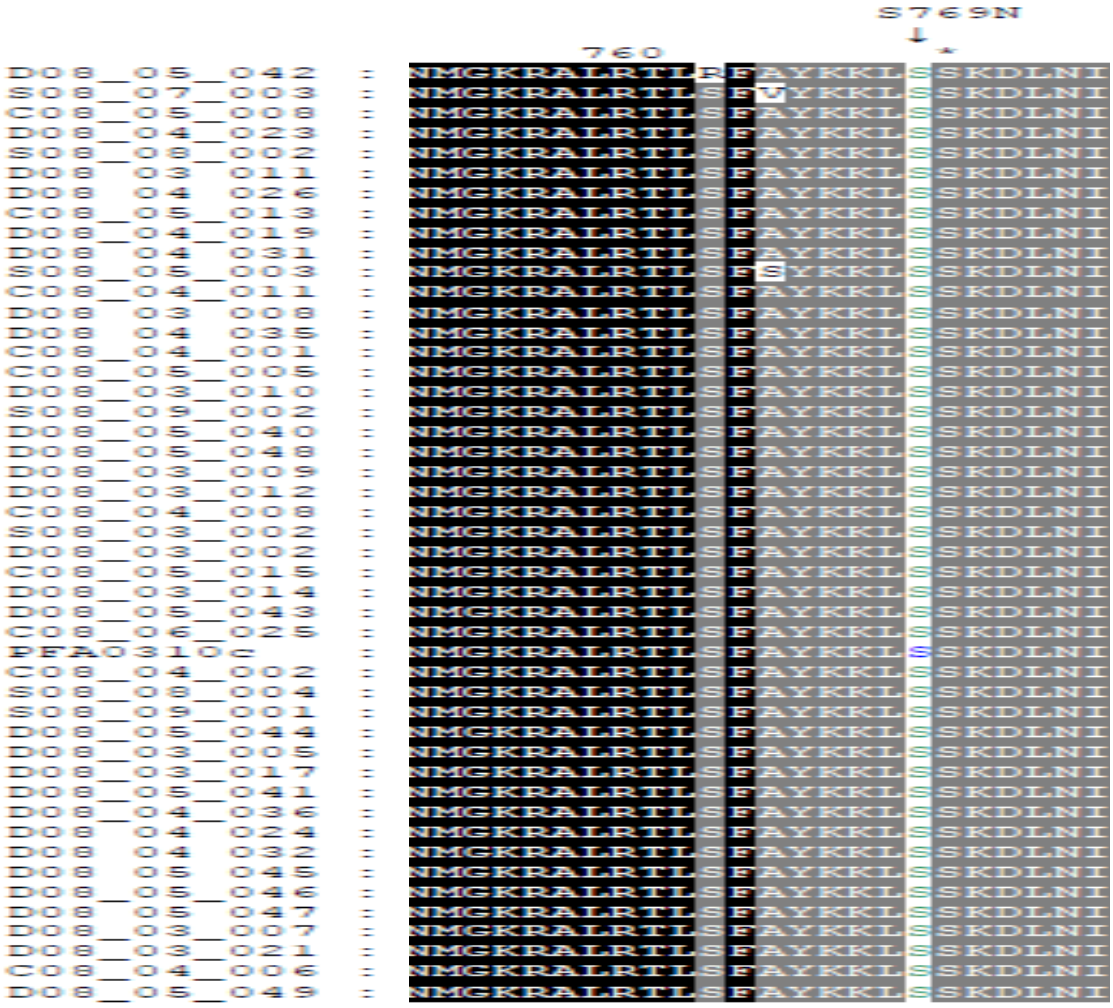

Figure 7a: Multiple sequence alignment of PfATPase6 amino acid sequences in the region around codon 769. Nucleotide sequences obtained from amplified gene fragments were translated into protein code and used to locate codons around position 769. The alignment was carried out using Muscle 3.6 program.

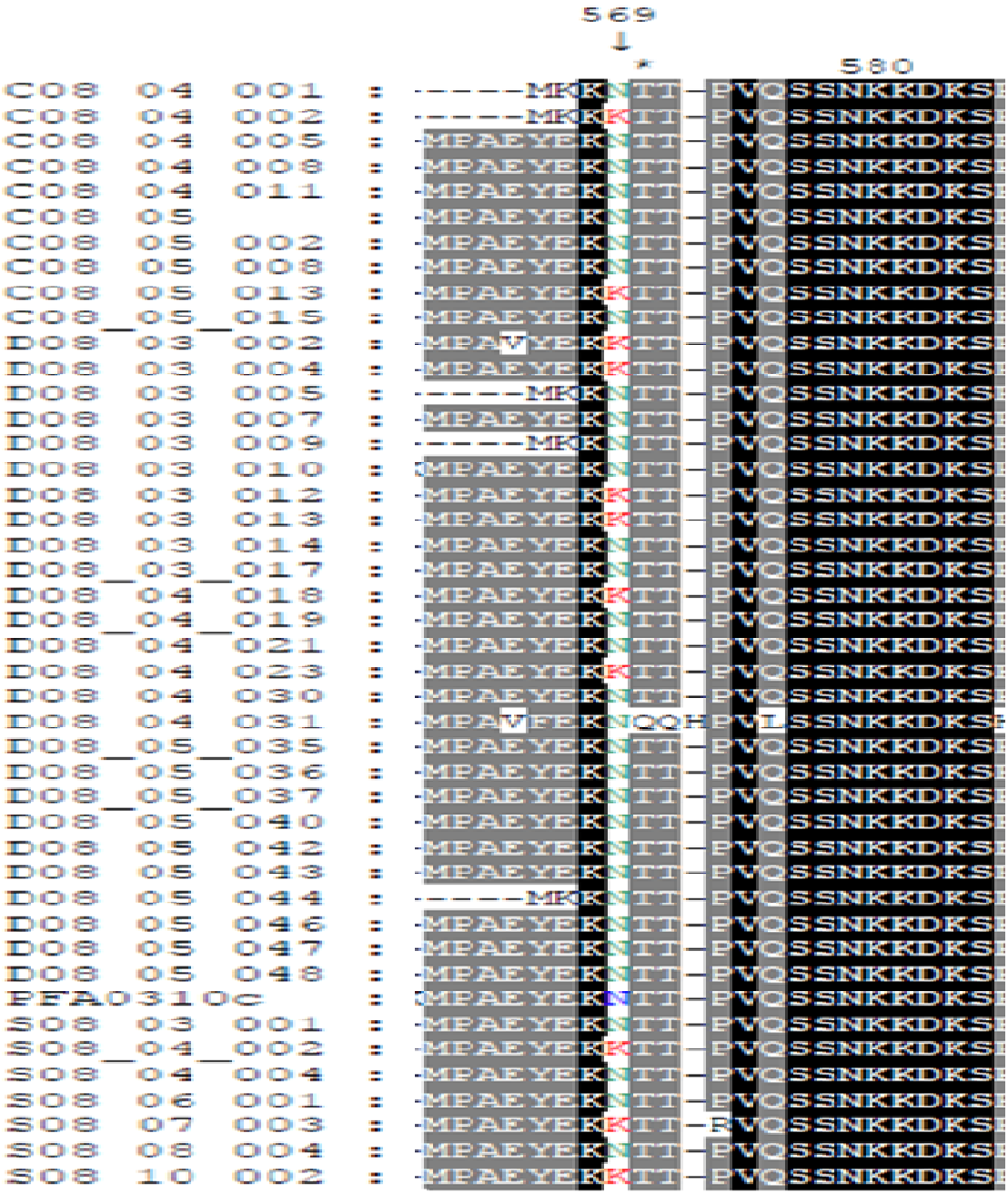

Figure 7b: Multiple Sequence Alignment of Amino acid Sequences of PfATPase6 to the 3D7 Clone focusing on codon 569. Nucleotide sequences obtained from amplified gene fragments were translated into protein code and used to locate codons that were affected. The alignment was carried out using Muscle 3.6 program focusing on SNPs affecting codons N569K.

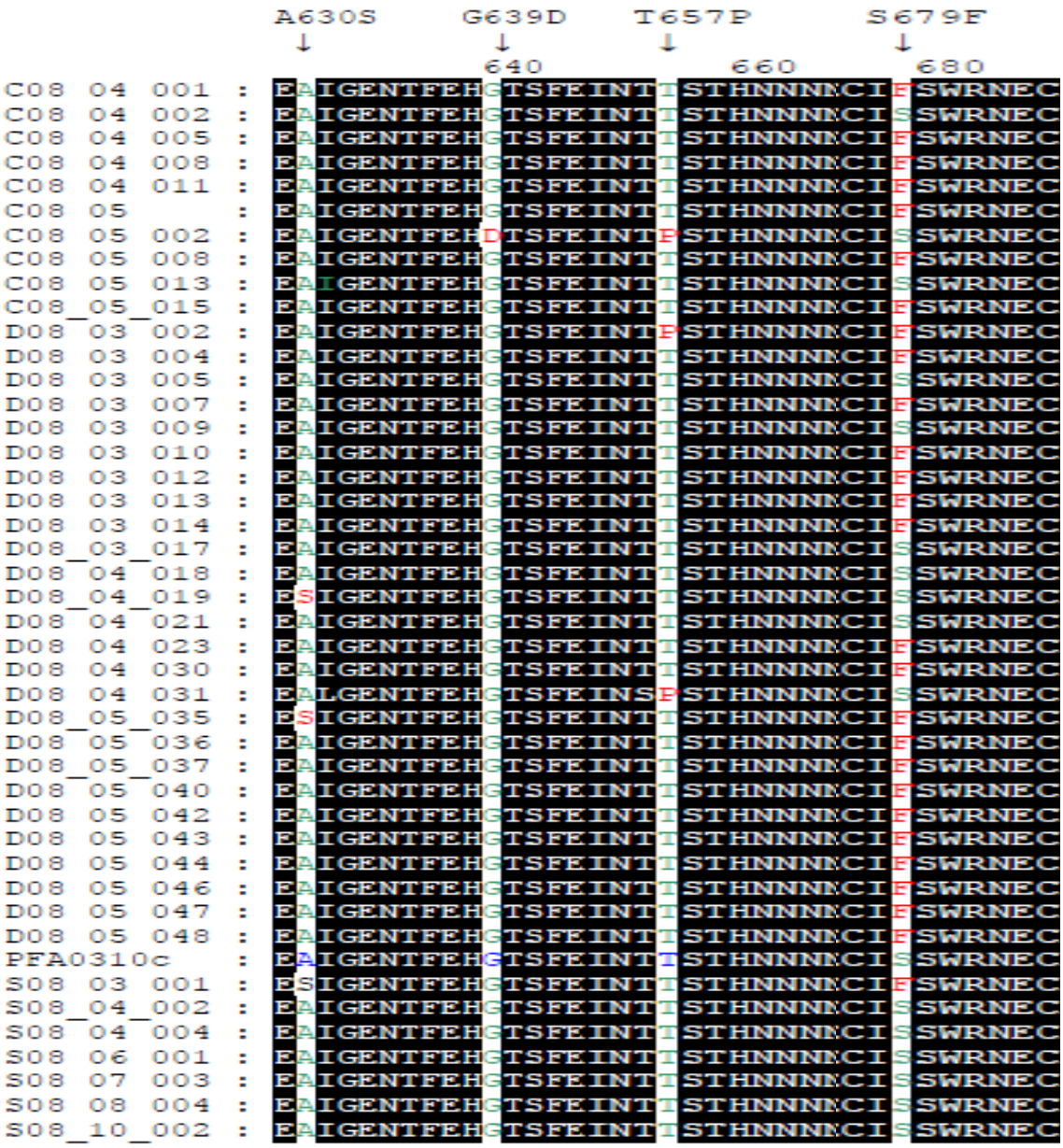

Figure 7c: Multiple Sequence Alignment of Amino acid Sequences of PfATPase6 to the 3D7 Clone focusing on codons 630, 639, 657 and 679. Nucleotide sequences obtained from amplified gene fragments were translated into protein code and used to locate codons that were affected. The alignment was carried out using Muscle 3.6 program focusing on SNPs affecting codons A630S, G639D, T657P and S679F.

CHAPTER FIVE

5.0 Discussion, Conclusion and Recommendation

5.1 Genetic Polymorphism and Artemisinin Resistance in the PfATPase6 genes

Among the parasites studied, single nucleotide polymorphisms (SNPs) were observed at positions 1707 (Tyrosine to Adenine), 1888 (Guanine to Tyrosine), 1916 (Guanine to Adenine), 1969 (Adenine to Cytosine) and 2036 (Cytosine to Tyrosine) [3D7 numbering system] in the PfATPase6 gene. These SNPs correspond to codons 569, 630, 639, 657 and 679 changing the amino acids at these positions as follows: N569K (Asparagine to a Lysine), A630S (Alanine to Serine), G639D (Glycine to Aspartate), T657P (Threonine to Proline) and S679F (Serine to Phenylalanine). All these mutations result in amino acid substitutions with radically different physiochemical properties that may dramatically alter the functionality of the enzyme. Thus, the changes at codons 569, 630, 639, 657 and 679 result into: polar uncharged to positively charged, non-polar aliphatic to polar uncharged, small non-polar to negatively charged, aliphatic polar to cyclic polar and polar uncharged to aromatic side chains respectively. Majority of these SNPs have been reported in field isolates of *P. falciparum* (Ibrahim, *et al*. 2009).

Interestingly, the SNP at nucleotide 2036 affecting codon 679, which was the most commonly observed mutation in the parasites in this study, have not been observed elsewhere before. This is therefore the first report of this unique mutation in field isolates of *P. falciparum* parasites. The observation that codon S679F had the highest incidence of 73.07% at Kisumu District hospital followed by Kericho District Hospital with 70% and

only at 12.5% for specimens collected from the Kisii District Hospital was an important observation. Since Malaria is endemic in Kisumu where Kisumu District Hospital is found and only sporadic and seasonal in Kisii district served by the Kisii District Hospital, this observation suggests that the S679F mutation is associated with endemic nature of *P. falciparum*. It may also suggest that this codon is an important marker that should be monitored as it may result in fitter parasites under the artemisinin drug pressure.

No mutations were detected at codons 89 and 769 among all samples analyzed in this study. This may indicate that these codons are not under selective pressure in the western region of Kenya. The mutations have also not been observed in China (Zhang, *et al*. 2008) and Tanzania where the mutation was assayed by either DNA-microarrays assays (Falade, *et al*. 2005) or by nucleotide sequencing (Menegon, *et al*. 2008). Whereas the PfATPaseS769N mutation has been described as a potential molecular marker for *P.falciparum* resistance to artemisinin (Jambou, *et al*. 2005) this mutation is very rare in African samples (Menegon, *et al*. 2008). It has only been found once by Cojean, *et al* 2006 (Uhlemann, *et al*. 2005) and may therefore not be an appropriate marker for resistance in Africa. Since none of the cases under this study portrayed preponderance for this mutation, it may be inferred that during the study period, there was no resistance to Artemisinin in western Kenya, if indeed the S769N change is associated with resistance. Therefore, absence of such a resistance marker in parasites in the western Kenya where malaria is endemic supports the Kenyan government's policy decision to adopt ACT as the first line treatment for uncomplicated malaria.

However, other PfATPase6 mutations were observed on the gene. These mutations were shown to occur repeatedly in the isolates at varying percentages. This study describes for the first time a PfATPase6 gene sequence polymorphism in Western Kenya. It increases the number of sample sequences and mutation points, adding two new mutation points: S679F and T657P. Among the five disparate mutations observed, only two, the N569K (25%) and S679F (61.36%), were frequent in the isolates. This observation supports results obtained in this region by Dahlstrom, *et al*, 2008, who also found a high prevalence of the N569K mutation in Zanzibar (36%) and Tanzania (29%). Furthermore, 7 mutations sites for 71 specimens from west, central and southeast Africa including Madagascar (Menegon, *et al*. 2008).

The frequently occurring mutations, S679F and N569K, were occurring together in 15.21% of the samples. However, *in vitro* drug susceptibility testing showed that the median of their IC_{50} values of samples with these mutations did not vary significantly with those samples that did not have these two mutations ($p>0.05$, 95% confidence interval), implying that there was no association between these two mutations and drug susceptibility/resistance.

Further analysis between IC_{50} values of the wild type isolates and the isolates having N569K revealed that the medians did not vary significantly as was the case for S679F and isolates having all the mutations. These results mean that the isolates from these three sites do not have any resistance for ACT for malaria treatment as a first line therapy.

Under the amplification conditions developed for the study, 2 samples could not be sequenced for codon S769N and 6 for codon N569K. This failure in sequencing of the 8 samples was attributed to low-level double sequences, which could have been due to the repetitive nature of the target sequence and possibly due to multi-priming effect.

5.2 Conclusions

A novel SNP at position 2036 (Cytosine to Tyrosine) of the PfATPase6 gene affecting codon 679 and converting Serine (a polar uncharged amino acid) to Phenylalanine (an aromatic amino acid) was discovered in majority (61.36%) of *P. falciparum* parasites circulating in Western Kenya. This discovery shows that the molecular diversity in the PfATPase6 gene is more pronounced than previously demonstrated and that the variable genetic background needed for the artemisinin-driven selection of resistant variants could be present in Africa. No S769N mutations were observed in this study. Since this mutation is presently a candidate as a molecular marker for artemether resistance, results from this study show that parasites that circulated in Western Kenya during the study period were all sensitive to artemisinin. Since the IC_{50} values were not significantly different ($p>0.05$ 95% C.I), the mutations do not have any effect on malaria treatment in Western Kenya using artemisinin.

5.3 Recommendations

A survey for N569K and S679F distinct PfATPase6 mutations should be conducted in other malaria endemic regions in Kenya, like the coast region so as determine the National prevalence rate of the mutation. This mutation may be used as a marker of *P. falciparum* parasites originating from this region

Even though it was not observed in this study, the PfATPase6 S769N mutation is a potential molecular marker for *P.falciparum* resistance to artemisinin (Jambou, *et al*. 2005). Thus, it should be continuously monitored for mutations in the malaria endemic areas in Kenya.

Future functional and structural studies, including PfATPase6 protein X-ray crystallography, three-dimensional determination of the protein, docking simulation and binding affinity, may be useful for prediction of the impact of the diverse mutations and the design of future molecular monitoring tools supporting the national control programmes. These studies may also lead to development of novel drugs that target the PfATPase6 protein for therapy against *P. falciparum* malaria.

At a time when almost all African countries have adopted artemisinin derivatives as first-line treatment for malaria, monitoring of the molecular evolution of PfATPase6, the key gene in artemisinin resistance, is of crucial importance. The baseline data established before the ACT implementation will support comparisons in future.

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Appendices

APPENDIX 1: Informed Consent Form.

PATIENT IDENTIFICATION NUMBER…….....

HEALTH FACILITY…….…….

ADDRESS……...……....

PHONE NUMBER…….

I, ……..., understand and accept to be involved in the study described above willingly and not in any kind of duress or bribery. Any bodily injury inflicted upon me shall be adequately compensated by the investigator.

PATIENT'S SIGNATURE……...……...

DATE…….……...

WITNESS' NAME……

SIGNATURE…….……

DATE…….……

INVESTIGATOR'S NAME…….

SIGNATURE…….……

DATE…….……

APPENDIX 2: Showing Data Analysis Results by Kruskal-Wallis test (Graph pad Prism 4 ®)

Table Analyzed			
Data 1			
Kruskal-Wallis test			
P value	0.2441		
Exact or approximate P value?	Gaussian Approximation		
P value summary	Ns		
Do the medians vary signif? (P < 0.05)	No		
Number of groups	6		
Kruskal-Wallis statistic	6.698		
Dunn's Multiple Comparison Test	Difference in rank sum	P value	Summary
Wild Type verses N569K	18.18	P > 0.05	Not Signi.
Wild Type verses T657P	28.05	P > 0.05	Not Signi
Wild Type verses S679F	15.19	P > 0.05	Not Signi
Wild Type verses N569K&S679F	14.66	P > 0.05	Not Signi
Wild Type verses ALL	28.05	P > 0.05	Not Signi
N569K verses T657P	9.875	P > 0.05	Not Signi
N569K verses S679F	-2.984	P > 0.05	Not Signi
N569K verses N569K&S679F	-3.518	P > 0.05	Not Signi
N569K verses ALL	9.875	P > 0.05	Not Signi
T657P verses S679F	-12.86	P > 0.05	Not Signi
T657P verses N569K&S679F	-13.39	P > 0.05	Not Signi
T657P verses ALL	0	P > 0.05	Not Signi
S679F verses N569K&S679F	-0.5339	P > 0.05	Not Signi
S679F verses ALL	12.86	P > 0.05	Not Signi
N569K&S679F verses ALL	13.39	P > 0.05	Not Signi

APPENDIX 3: GenBank Accession Numbers for the Nucleotide Sequences:

HQ259321
HQ259322
HQ259323
HQ259324
HQ259325
HQ259326
HQ259327
HQ259328
HQ259329
HQ259330
HQ259331
HQ259332
HQ259333
HQ259334
HQ259335
HQ259336
HQ259337
HQ259338
HQ259339
HQ259340
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HQ259372
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HQ259380
HQ259381
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HQ259402
HQ259403
HQ259404
HQ259405
HQ259406
HQ259407
HQ259408
HQ259409

APPENDIX 4: KEMRI SSC 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: kemri-hq@nairobi.mimcom.net; director@kemri.org; Website: www.kemri.org

ESACIPAC/SSC/3011 **14th October, 2008**

Douglas Walsh

Thro'
Director, CCR
NAIROBI

22/10/08

REF: SSC No.1330 (Amendment) - Epidemiology of Malaria and Drug sensitivity patterns in Kenya

I am pleased to inform you that the above mentioned proposal in which you are the PI, was approved for implementation by the KEMRI Scientific Steering Committee (SSC), during its **150th SSC** meeting held on **7th October, 2008** and has since been forwarded to the Ethical Review Committee (ERC) for consideration.

The SSC however, advises that work on this project can only start when ERC approval is received.

C. Mwandawiro, PhD
SECRETARY, SSC

APPENDIX 5: KEMRI ERC Approval

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840 - 00200 NAIROBI, Kenya
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E-mail: kemri-hq@nairobi.mimcom.net; director@kemri.org; Website: www.kemri.org

KEMRI/RES/7/3/1 **NOVEMBER 5th, 2008**

FROM: **SECRETARY, KEMRI/National Ethical Review Committee**

THRO': **DR. RJ RASHID,**
CENTRE DIRECTOR, CCR,
NAIROBI.

Forwarded 7/11/08

TO: **DR. DOUGLAS WALSH, (PRINCIPLE INVESTIGATOR)**

RE: **SSC NO. 1330 (AM): EPIDEMIOLOGY OF MALARIA AND DRUG SENSITIVITY PATTERNS IN KENYA (v. 6 dated 25 August 2008)**

Dear Sir,

This is to inform you that during the 160th meeting of KEMRI/National Ethical Review Committee held on 4th NOVEMBER 2008, the requested amendments to the abovementioned study were reviewed.

We acknowledge receipt of several amendments to the above protocol occasioned by the Walter Reed Army Institute of Research (WRAIR) Institutional Review Board (IRB) review.

The requested amendments do not alter the design of the study nor do they alter the risk to the study participants and are therefore granted approval. You may continue with your study.

Respectfully,

RCKithinji

R. C. KITHINJI,
FOR: SECRETARY,
KEMRI/NATIONAL ETHICAL REVIEW COMMITTEE