COMPARISON OF THE EFFECTS OF A FORMULATED TRANSPORT MEDIUM AND EDTA ANTICOAGULANT ON *IN VITRO* ANTIMALARIAL DRUGS ACTIVITY AGAINST *PLASMODIUM FALCIPARUM* STANDARD CLONES AND FIELD ISOLATES

By

SCOLASTICA C. KORIR

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DECLARATION

I declare that this thesis is my original work and has not been submitted wholly or in part for any award in any institution.

Scolastica Chepngetich Korir

PG/PHD/00053/2013

Signature _____ Date: _____

Supervisors

This thesis is submitted for examination with our approval as \university supervisors.

1. Prof. Rose Kakai Signature …….. Date ……..….

   Department of Medical Microbiology,

   School of Medicine.

   Maseno University

2. Dr. Hoseah Miima Akala Signature …….. Date ……..….

   Kenya Medical Research Institute / Walter Reed Project

   Malaria Drug Resistance Project,

   Kisumu.

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DEDICATION

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ABSTRACT

Malaria is an important public health problem world wide with 283 million infections resulting in 584,000 deaths per year globally. In Kenya, malaria accounts for an estimated 18% of outpatients and 6% of hospital admissions. Antimalarial resistance is among the contributory factors to an increase in mortality. Kenya was the epicenter of chloroquine resistance in Africa. Recent reports of reduced susceptibility to the commonly used artemisinin combination drugs at the Kenyan coast and other African countries indicate an urgent need for intensified surveillance of antimalarial drug efficacy. *In vitro* sensitivity testing is one of the preffered methods of measuring susceptibility. This method is able to measure *P. falciparum* susceptibility to several antimalarial drugs simultaneously, away from the influence of host immune related factors. The technique is being transitioned to *ex vivo* requiring tests on fresh sample. Such *in vitro* studies of field *P. falciparum* have been attributed to diminished viability as they transition from host ecosystem to lab conditions due to lack of a proper medium to stabilize the parasites outside the human host. It is therefore imperative to calibrate the sample stabilization media to reduce artificial effects to the assay.The objective of this study was to evaluate the effect of a formulated transport medium (TM) on viability of *Plasmodium* being transported to the lab for testing within 6 hours. Specifically the study assayed standard clones, field isolates and finally compared the results with other published findings to come up with antimalarial susceptibility profile of the region. Inhibition concetratin 50% (IC_{50}) for field isolates transported in TM that is less costly was compared with that of Ethylene Diamine Tetra Acetic acid (EDTA) anticoagulant which allows better preservation of cellular components and morphology of blood cells for a longer period. This was a cross-sectional laboratory based epidemiological study. Blood samples were collected from 322 assenting individuals from Maseno division visiting Chulaimbo Sub county hospital and confirmed positive for malaria, under the approved protocol (accreditation number 01713). Each sample, split in to EDTA vs TM was analysed for susceptibility to artemether (ART), lumefantrine (LUM), dihydroartemisinin (DHA) and piperaquine (PPQ) using Malaria SYBR Green Assay. IC_{50} was determined for each sample between TM and EDTA using dose response curves. Mann Whitney test for comparing the medians IC_{50} values and Pearson correlation coefficient for establishing correlations of logarithimic values of IC_{50} for different drugs were used. In total 322 samples yielded paired results for ART, LUM, DHA and PPQ. The reference clones W2 (considered chloroquine resistant, mefloquine and artemisinin sensitive) and 3D7 (considered chloroquine and mefloquine sensitive) were used as internal controls. Results showed that the IC_{50} values of the field isolates in EDTA were higher although not significant (P=0.99, 0.74, 0.68, 0.82 for ART, LUM, DHA and PPQ respectively) than those in the TM. Among the clones, PPQ was the only drug with a high significant IC_{50} decrease (P<0.001) in TM for the W2 and a moderately significant decrease (P=0.028) in EDTA for 3D7 clone, while other drugs were not significant. These suggest a similarity in the two anticoagulants in preservation of the blood samples. Significant correlation was observed between DHA and ART (r=0.123, *p*=0.026); LUM (r=0.138, p=0.012) and PPQ (r=0.128, p=0.02), suggesting a possible cross resistance between the drugs. In conclusion, the clones and the field isolates in EDTA showed no significant difference results with those in TM. Despite the stabilizers exposure, field isolates' IC_{50} values generated in all the drugs were comparable with those found earlier for clinical isolates collected in other endemic areas. Lower IC_{50} values recorded by the field isolates against the antimalarials were indicative of their high susceptibility to the drugs. The use of TM could help on real time interventions leading to detection of parasites resistance at its onset thus reducing mortality from malaria related cases. However, further research should be done to find out the practical time TM can keep the parasites viable.

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LIST OF ABBREVIATIONS

ACD	Acid Citrate Dextrose
ACT	Artemisinin Combination Therapy
ANOVA	Analysis of Variance
ART	Artemether
CM	Complete medium
CMS	Complete Medium with Serum
CQ	Chloroquine
DHA	Dihydroatermisinin
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene Diamine Tetra Acetic Acid
ELISA	Enzyme linked Immunosorbent Assay
G6PD	Glucose 6 Phosphate Dehydrogenase
HRPII	Histidine rich protein II
HEPES	Hydroxy Ethyl Piperazineethane Sulfonic
IC_{50}	Inhibitory concentration $_{50}$
IM	Incomplete medium
KEMRI	Kenya Medical Research Institute
LUM	Lumefantrine
MOH	Ministry of Health
MSF	Malaria SYBR Green 1 Fluoresence Assay
NACL	Sodium Chloride
$NAHCO_3$	Sodium Hydrogen Carbonate
PBS	Phosphate Buffered Saline

PCV	Packed cell volume
Pfcrt	*Plasmodium falciparum* chloroquine resistant transporter
Pfmdr	*Plasmodium falciparum* multidrug resistant gene 1
pLDH	Parasite Lactate Dehydrogenase
PPQ	Piperaquine
RBCS	Red Blood Cells
RPM	Revolutions per minute
RPMI	Roswell Park Memorial Institute
SIT	Schizont inhibitory technique
SP	Sulfadoxine pyrimethamine
TM	Transport medium
WHO	World Health Organisation

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DEFINITION OF TERMS

50% inhibitory concentration: The concentration of a drug that gives half maximal response.

Drug resistance: Ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those recommended but within tolerance of the subject.

***In vitro*:** Experimental studies or biological process made to occur in a laboratory vessel or other controlled experimental environment rather than within a living organism or natural setting.

In vivo: Experimental studies or biological process made to occur within a living organism or natural setting.

Mixed infections: An infection containing more than one type of parasite species or genotype.

Parasitaemia: Measurement of parasite load in an organism or presence of parasites in circulating blood.

Pharmacoepidemiology: The study of the uses and effects of drugs in well defined population.

CHAPTER 1.0: INTRODUCTION

1.1 Background Information

Malaria remains a major public health problem in many countries of the world. Despite the progress in reducing malaria cases and deaths, it is estimated that 214 million cases of malaria occur worldwide, leading to approximately 438 000 malaria deaths. Most of these deaths occur in the African Region (90%), followed by the South East Asia Region (7%) and the Eastern Mediterranean Region (2%). The African Region accounts for most global cases of malaria (88%), followed by the South-East Asia Region (10%) and the Eastern Mediterranean Region (2%) (WHO, 2015).

The global burden of mortality predominantly rests on countries in sub-Saharan Africa. About 313 million people in the 12 countries of the subregion are at some risk for malaria, with 254 million at high risk amounting to 80% of the deaths (Murray *et al.*, 2014). It is the leading cause of death and disease with young children and pregnant mothers mostly affected (WHO, 2013). By 2015, the under five motality rate was estimated to be 82 deaths per 1000 live births, leading to 2.9 million deaths annually (WHO, 2015).

In Kenya Malaria is among the leading cause of morbidity and mortality (Kenya Ministry of Health, 2015). In endemic zones, there was an increased in malaria incidence towards December 2013 from 4.6 to 8.7 cases per a thousand people (Kenya Ministry of Health, 2013). However, in 2014 a higher incidence of malaria was witnessed though with a steady decline towards December from 11.7 to 9.7 cases per a thousand people (Kenya Ministry of Health, 2014). This still renders malaria the leading tropical parasitic disease becoming one of the reasons hindering economic development among African nations.

Efforts on preventive measures and availability of effective therapies have not been successful in controlling malaria (Nguessan *et al.,* 2007). Uses of insecticides impregnated and treated nets, indoor residual spraying, environmental modifications, have faced setbacks in the recent years (Vulule *et al.,* 1999;; Yewhalaw *et al.,* 2010). Compexity of the *Plasmodium* parasite and its lifecycle (Gardner *et al.,* 2002), poor understanding of the interaction between the parasite and the immune system (Langhorne *et al.,* 2008) and extensive antigenic variation (Scherf *et al.,* 2008) have hampered vaccine development efforts.

Antimalarial drugs, in combination with mosquito control programs, have historically played a key role in controlling malaria in endemic areas, resulting in significant reduction of the geographic range of malarial disease worldwide (Florens *et al.,* 2002). However, the continued emergence and spread of multi drug-resistant strains of *Plasmodium falciparum* are arguably one of the most pressing problems today (Bennett *et al.,* 2004) hence turning back the clock on control efforts (WHO, 2010).

The major contributory factor to antimalarial resistance has been documented to have been contributed by the inappropriate supply of antimalarials due to unregulated market, selfmedication and underdosing (Sammy *et al.,* 2013). Resistance to antimalarials, and lately reduced susceptibility to the commonly used artemisinin combination drugs at the Kenyan coast and other African countries (Borrmann *et al.,* 2011), indicate an urgent need for surveillance to monitor sensitivity profiles of antimalarial drugs across Africa. To accommodate this reality, a faster, effective, less expensive, and high through put means of screening the activities of drugs against a variety of malarial parasites would greatly assist in drug surveillance, pharmaoepidemiology and preclinical drug development. Currently, available methods are costly and highly dependent on culturing of parasites prio to drug tests. Culturing alters the composition

of the parasite since evolution is continuous, therefore the characteristics of the initial genotype will not be shown.

Studying drug resistance in *Plasmodium falciparum* requires accurate measurement of parasite response to a drug. Factors such as mixed infection of drug resistant and sensitive parasites can influence drug test outcome (Liu *et al.,* 2008). The parasites of *P. falciparum*, as well as those of other plasmodial species, exhibit a high degree of biological and pathological diversity (Brumpt, 1999) between strains with respect to a number of important characteristics, such as pathogenicity, responses to drug treatment, and transmissibility by mosquitoes.

Further, each *Plasmodium* species comprise a number of distinct strains which are autonomous, have stable biological entities and distinguishable by clinical, epidemiological or other features (Elis *et al.,* 2008). In addition, any strain infecting a host at any one time may often comprise of several distinct populations. As a result, considerable diversity could be demonstrated in the parasites present at a given geographical location (Mackinnon *et al.,* 2009).

The exact nature of the mechanisms underlying most of the parasite's variable characteristics includes pathogenicity and transmissibility by mosquitoes. Therefore, to guide on the range of sensitivities and reduce such variations, it is important to assay reference clones since they have reduced biological variability due to the absence of resistant and sensitive subpopulations of the parasites (Liu *et al.,* 2008). Clones 3D7 and W2 are standard reference clones for *P. falciparum* which are susceptible and resistant to antimalarials respectively among other clones.

Drug efficacy in the field has been performed *in vivo* defined as "experimental studies occurring within an organism or in a natural setting". This has been achieved by regular measurement of body temperature and microscopic examination of blood films of the subjects (Noedl *et al.,*

2003). This method has been considered the "gold" standard for drug resistance since it takes in to account host parasite interactions. However, it is costly and logistically complex in countries that have a low moderate intensity of transmission with limited access to malaria endemic areas. Additionaly, test are affected by variables like host immune status, diet, other drugs or intestinal parasites that might be out of control (Basco, 2003).

Challenges associated with the assessment of antimalarial drug resistance *in vivo* led to the introduction of a number of *in vitro* tests for the measurement of antimalarial drug susceptibility in the late 1970s (Rieckmann *et al*., 1978). *In vitro* tests avoid many of the confounding factors which influence *in vivo* tests by removing parasites from the host and placing them into a controlled experimental environment. Thus may be a more objective method to detect drug resistance since it eliminate these host factors, including reinfections, immunity, pharamacodynamics, and pharmacokinetics (Wirjanata *et al.,* 2015). *In vitro* techniques allow multiple tests to be performed on isolates, several drugs to be assessed simultaneously, and experimental drugs to be tested (Bloland, 2001).

There are many *in vitro* testing techniques endorsed by the World Antimalarial Drug Resistance Network in 2007: The WHO Microtest (WHO, 1990), the radioisotope hypoxanthine incorporation (Desjardins *et al.,* 1979), *Plasmodium* lactate dehydrogenase immune detection (Kaddouri *et al*., 2006). Lately, histidine-rich protein II assays (Noedl *et al*., 2005) and SYBR Green I (Smilkstein *et al.*, 2004).

The WHO Microtest (WHO, 1990) is inexpensive but particularly labor-intensive and subjective due to variations in the expertise of the microscopists responsible for reading the slides. The technique therefore becomes problematic and impractical as volume of samples increase (De Monbrison *et al.,* 2003).

The isotopic assay is widerly published and accepted, however, this method is time-consuming and the need for the disposal of radioactive materials has limited its widespread use, especially in countries that cannot afford the cost of a liquid scintillation counter. Reagents are difficult, if not impossible, to obtain (Bacon *et al.,* 2007).

Two enzyme-linked immunosorbent assay (ELISA)-based methods that use monoclonal antibodies to *Plasmodium* species or plasmodial lactate dehydrogenase (pLDH) (Druilhe *et al.,* 2001)) and histidine-rich protein II (HRPII) (Noedl *et al.,* 2005) have begun to be widely used in recent years due to their ease of use. However, the test kits can be difficult to obtain, and the performance of these multiple-step assays is time-consuming (Noedl *et al.,* 2002).

Fluorescent-based techniques that use SYBR green I dye to determine parasite growth following *in vitro* drug susceptibility assay have been described (Bennett *et al.,* 2004). The fluorescence-based method is a simple, convenient, one step procedure that is relatively rapid and reproducible. The dye is readily available from multiple sources worldwide. The technique is less costly than radioisotope assays (Smilkstein *et al.,* 2004) and can also be used in high parasite densities (Bacon *et al*., 2007). The assay is exceptionally fast since one reagent is added to the plate after parasite growth and requires no washing and filtering steps (Bennet *et al*., 2004).

These *in vitro* drug assays in the field are preferred and perfomed on fresh isolates immediately after collection (Basco, 2004) due to simplicity to perform than culture adapted (Akala *et al.,* 2011; Bacon *et al.,* 2007). However, blood samples containing *P. falciparum* parasites for testing may be stored at collection sites for days or transported long distances to the laboratory before cultivation (Akala *et al.,* 2011), a factor which could result in reduced viability during subsequent culture adaptation process. It has been reported that fresh isolates may not grow well initially in an *in vitro* culture, and some parasites eventually fail to establish during

culture because of problems of adapting and surviving *in vitro* culture conditions, (Liu *et al.*, 2008). It is important to come up with a medium which can keep the parasites energized and viable while reaching the laboratory to enhance immediate establishment of drug tests.

Normally samples for *in vitro* study are collected from consenting patients with *Plasmodium* infection in a hospital set up, but due to lack of infrastructure to perform the experiments at the field sites and hospitals, there is need to transport these samples to a distant laboratory. Any considerable delay in performing the assay, from 24 hours and beyond, results in a decrease in parasite viability accompanied by decreasing IC_{50}s values obtained. This introduces an important bias and rendering more difficult current attempts by several research groups to standardize *in vitro* assay methods (Basco, 2004).

Different artificial media have been devised to enhance immediate survival, prolonged survival and growth of the parasites outside the human body (Freshney, 2005). The important step in *Plasmodium* culture is the ability to stabilize the parasites once outside the natural host. A stabilizer provides a "physiological" or balanced salt solution, to maintain proper pH, ideal osmotic pressure, and provide a source of energy for parasites. This environment tries to reduce the shock experienced, which can lead to death due to the abrupt change of environment. (Freshney, 2006).

To shed light on the significance of a nourishing medium to *Plasmodium falciparum* infected blood cells, this study used a formulated transport media (TM) which is less costly to stabilize and transport samples to the laboratory alongside EDTA anticoagulant tubes ($EDTAK_3$ 4ml) and compared *in vitro* response of the isolates transported in either of the reagents. EDTA anticoagulant has the advantages of a lack of inhibitory action on DNA allowing the best

preservation of cellular components and morphology of blood cells for a longer period (Reardon *et al.*, 2003), thus best for hematologic and biochemical investigations (Basco, 2004). However

1.2 Statement of the problem

There is a growing preference of *ex vivo/in vitro* antimalarial drug assay techniques. The main reasons for reliance on this technique are that it is practical, scallable; a large number of field isolates can be rapidly and inexpensively collected and analyzed. However, there is lack of a solution that stabilizes and nourishes parasites post collection therefore, delaying immediate establishment of drug tests. Currently, available solutions are costly and highly dependent on culturing of parasites prior to drug tests. Culturing alters the composition of the parasite due to adaptation selection; therefore making it hard to decipher the characteristics of the initial genotype.

Inappropriate use due to selfmedication, underdosing, non completion of dosage and unregulated market for antimalarials emerge a major contributory factor to resistance (Sammy *et al.*, 2013). This behaviour is common in Kisumu and rural western Kenya (Geisler *et al.*, 2000) where Maseno division the study site is located. This calls for the implementation of a sustainable, less costly tool for drug surveillance and pharmacoepidemiology. The newer *in vitro* testing techniques like the SYBR Green 1 offers a robust platform for depiction of drug performance because it is an exceptionally fast, simple, one step procedure whose results are rapid and reproducible (Rengarajan *et al.*, 2002). It also discerns drug IC_{50}s with *P. falciparum* parasitemias as low as 0.112%, (Akala *et al.*, 2011; Johnson *et al.*, 2007, Cheruiyot *et al.*, 2016). However, its measure of sensitivity or resistance entirely depends on the growth of parasites when subjected to the antimalarials (WHO 2001) in an artificial environment. Studies have reported failure of *P. falciparum* growth attributed to impaired viability due to a prolonged time between the times when the samples were collected and when they were tested (Basco *et*

al., 2004). These imply that there is poor adaptation of parasites once removed from the human host thus may not grow when cultured. For immediate survival, prolonged survival and growth, *Plasmodium* requires a proper transition from the natural host to an appropriate conducive medium especially for transportation to the laboratory. Therefore, there is need for parasite collection/stabilization in a transport medium (TM).

1.3. Objectives of the study

1.3.1. General objective

To compare the effects of a formulated transport media and EDTA anticoagulant on *in vitro* antimalarial drugs activity against *Plasmodium falciparum* standard clones and field isolates.

1.3.2. Specific objectives

1. To establish the *in vitro* responses of *P. falciparum* 3D7 and W2 reference clones incubated in TM and EDTA for 3 to 6 hours to artemether, lumefantrine, dihydroartemisinin and piperaquine.
2. To determine the *in vitro* responses of *P. falciparum* field isolates transported in TM and EDTA to artemether, lumefantrine, dihydroartemisinin and piperaquine.
3. To determine the susceptibility profile of the population in Maseno Division to artemether, lumefantrine, dihydroartemisinin and piperaquine by correlating the IC_{50} responses for samples transported in TM and EDTA anticoagulants.

1.4 Research Questions

1. What is the *in vitro* response of *P. falciparum* 3D7 and W2 reference clones in EDTA and TM to artemether, lumefantrine, dihydroartemisinin and piperaquine.
2. What is the *in vitro* response of *P. falciparum* field isolates in EDTA and TM from Chulaimbo Sub District hospital to artemether, lumefantrine, dihydroartemisinin and piperaquine.

3. What is the susceptibility profile of the pupolation in Maseno division to artemether, lumefantrine, dihydroartemisinin and piperaquine?

1.5 Justification of the Study

The WHO malaria report 2015 urges on the real-time detection of parasite resistance in order to improve interventions. For the case of *in vitro/ ex vivo* antimalarial drug testing, this goal; is impeded by lack of suitable medias to stabilize blood samples. Fresh clinical isolates are preferred since they overcome the laboratory induced artifacts and give true values for the parasite population obtained from infected patients. Therefore, there is need to sustain the viability of these isolates while reaching the laboratory. In that regard this study validated the efficacy of a formulated transport medium for stabilizing malaria positive blood samples prior to *ex vivo* drug testing.

Standard clones in the formulated media and conventional medium were tested in parallel. Formulated media and conventional medium were also used for stabilizing field isolates during transportation to the lab. IC_{50}s of field isolates and reference clones stored in either of the stabilizers were determined and compared. **Finally comparison of the clones and the field isolates was done with other published studies to** establish susceptibility profile. The TM will ease real-time sensitivity testing of parasites in natural infection allowing accurate readouts, prior to culture-adaptation. Use of TM in stabilizing isolates for drug sensitivity testing would give insights on the use and effects of drugs (pharmacoepidemiology), and provide a quick way of detecting resistance and prevents malaria related deaths. It would also improve public knowledge on the parasite, parasite biology knowledge, and be used on preclinical drug development.

1.6. Limitations of the study

Some of the limitations faced by the study were: variation on the estimation of parasitaemia, possibility of drugs residues despite washing by centrifugation, possibility of the effects of the media the standard clones had been exposed to before subjecting them to TM

CHAPTER 2.0: LITERATURE REVIEW

2.1 Introduction

This chapter gives a detail comparison, critique and knowledge gap on the literature for all the objectives; this has been discussed in subtitles including, malaria as a public health problem, drug resistance, *in vivo* and *in vitro* drug assay techniques, culturing *of Plasmodium falciparum*, proposed transport medium and the need for antimalarial sensitivity study.

2.2 Malaria as public health problem

Malaria is the world's most important parasitic infection and among the major health and developmental challenge for the poor countries of the world (Sachs and Malaney, 2012). Most malaria deaths and large proportion of morbidity are caused by *Plasmodium falciparum*. Malaria occurs in over 90% countries worldwide, 36% of the global population lives in areas where there is risk of transmission (WHO, 2013). *Plasmodium vivax,* although it rarely causes death, is a constant annoyance and the leading cause of morbidity due to the dormant phases of the parasite that reside in liver hepatocytes (Hyde, 2002).

In Kenya Malaria is still the leading cause of morbidity and mortality (Kenya Ministry Health 2014). The disease accounts for 30 percent of Kenya's outpatient visits, 19 percent of hospital admissions, two to three percent of inpatient deaths, and is a leading cause of death among children under the age of five (Kenya Ministry of Public Health and Sanitation 2012). Specifically, in Western Kenya, 32% of all deaths are associated with Malaria, It causes 22% deaths among children with severe malaria, out of which 10% occur in hospitals and 13% outside hospitals within 8 weeks after admission (Kenya Ministry of Public Health and Sanitation 2011).

Also, treatment of malaria with chloroquine in the region was associated with 33% case fatality rate compared to 11% for children treated with more effective regime (Jane *et al*., 2012).

A variety of drugs have been used to treat malaria in Kenya over the years, including quinine and related compounds such as chloroquine, antifolate combination drugs, antibiotics, and artemisinin compounds. Efforts to control the spread of the disease, however, are hampered by a growing trend of resistance to antimalarial medications. This is a great concern to the Kenyan government, whose goal is to decrease morbidity and mortality due to malaria by 30 percent by 2017 (Kenya Ministry of Public Health and Sanitation 2011). It is predicted that clinical infections and death will begin to increase due to rapid spread of drug resistance parasites (Hastings and Alessandro, 2000).

In addition to resistance to antimalarial combination therapies, there is also a concern with *Plasmodium* mixed infections. A mixed infection is defined as an infection with more than one type of species or genotype of *Plasmodium* (Ferdig and Su, 2000). Other groups have noted that a number of malarial infections are heterogeneous in their composition (Lorenzetti *et al.*, 2008). Mixed infections of different genotypes are highly prevalent in malaria-endemic areas, particularly in Africa and Southeast Asia (Kobbe *et al.*, 2006). In fact, almost all infections occurring in nature are mixed. Although highly understudied, the implications of a mixed infection are profound (Postigo *et al.*, 1998). They can cause a relapse as a result of emergence of the resistant subpopulation of parasites after the sensitive subpopulation has been eradicated by drug therapy.

The existence of a resistant population may be a result of both divergent evolutions, where parasites have acquired resistance mechanisms, and/or two cohabitating parasites when the individual is infected (Hyde, 2002). This phenomenon has been observed in areas of malaria

endemicity in Africa and Southeast Asia where the mixed-infection prevalence is as high as 30% (Mayxay *et al.*, 2004). However, there has been conflicting evidence as to the true frequency of *Plasmodium* mixed infections (Richie, 1988). Furthermore, this problem is confounded by the inability to properly identify and differentiate *Plasmodium* mixed infections. This phenomenon is possibly due to the assay's detection limit, where susceptibility of one population ceases to affect the profile of the other subpopulation, effectively masking the existence of that subpopulation. At this low parasite subpopulation level, dominance of a resistance mechanism may not be high enough in the population and may result in the appearance of only one phenotype. The reverse is also possible, where a large susceptible subpopulation may influence the apparent phenotype of the whole population.

Most naturally occurring malaria infections are composed of mixed subpopulations with different drug susceptibilities (Postigo *et al.*, 1998). It is likely that mixed infections of both drug sensitive and resistant parasites in a patient will produce test readings not representative of a typical resistant or sensitive parasite. Thus, there is a need to identify these drug resistant sub populations to prevent treatment failures and misidentification of a populations susceptibility profile (Zimmerman *et al.*, 2004). This is always done by assaying reference clones in any *in vitro* study to get the range of sensitivities expected for the field isolates to reduce variations. Compared to the isolates, clones have reduced biological variability due to the absence of subpopulations of resistant and sensitive parasites (Liu *et al.*, 2008).

2.3 Drug resistance

Antimalarial drug resistance has been defined as the "ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher

than those usually recommended but within tolerance of the subject (WHO, 2001). It has emerged as one of the greatest challenges facing malaria control today; it is a major obstacle to the successful treatment and control of malaria. It has been implicated in the spread of malaria to new areas and in reemergence of malaria in areas where malaria had previously been eradicated, but above all it has been identified as a factor of economic constraints for malaria control (Bloland, 2001). Due to the financial restrictions in the fight against the resurgence of malaria, the considerate use of the limited available resources is essential.

Chloroquine resistant *Plasmodium falciparum* has been reported in all Sub-Saharan African countries (WHO, 1999). In a previous *in vitro* study conducted in Younde results showed that about 50-60% of *Plasmodium falciparum* were resistant to chloroquine both *in vivo* and *in vitro* (Louis *et al*,. 1992). In Kenya Chloroquine (CQ) resistance was first reported in 1977 (Fogh *et al.,* 1979) and in 1993 resistance level had reached 70% among the population (Anabwani *et al.,* 1996). This led to changing of the first line treatment from CQ to sulfadoxine – pyrimethamine (SP Fansidar) (Shretta *et al.,* 2000) by the Kenya Ministry of Health (MOH). In 2004, malarial parasites resistance towards SP was also detected and due to further resistance, the MOH changed the first line of drug to Arthemeter / lumefantrine (Coartem) ((Amin *et al.,* 2007) which being used to date.

However, reports have shown that the drug has more recent observation of reducing responses rates to ACTs on the Kenyan coast (Borrmann *et al.,* 2011) and the emergence of artemisinin resistance in South East Asia, (WHO, 2007; Maude *et al.,* 2009; Noedl *et al.,* 2010) where besides a delay in parasite clearance, the *ex vivo* sensitivity of *P.falciparum* to artemisinin derivatives has declined substantially over the last few years. Such high levels of resistance to antimalarials have been registered in many malarial zones of the world such as Papua New

Guinea, 85% (Al Yaman *et al*., 1996); Tanzania, 44% (Ekvall *et al.,* 1998) and Burundi Highlands, 77.8% (Di Perri *et al*., 1998). This resistance has frequently been observed after massive intake of antimalarial medicaments for prophylaxis (WHO, 1996).

Outside Africa, the same resistance has been experienced, in Colombia where clinical efficacy studies of antimalarial drugs have shown therapeutic failures of up to 90% in patients with uncomplicated *Plasmodium falciparum* malaria treated with CQ. This evidence was the basis for changing the national antimalarial drug policy in 1999, when CQ was replaced by amodiaquine (AQ) combined with sulfadoxine/pyrimethamine (SP) as the treatment of choice (MS 1999). Similarly, therapeutic failures to AQ reached up to 50% in the Pacific coast region and therapeutic failures to SP also reached up to 87% in the Amazon Region (Osorio *et al.,* 2007).

Resistance to alternative drugs, such as amodiaquine, sulfadoxine / pyrimethamine, quinine, and mefloquine, exists to a varying degree in some endemic regions (Wernsdorfer, 1991).

One of the underlying factors that may favor the development of drug resistance is cross-resistance among chemically similar drugs (Wernsdorfer and Payne, 1991). The currently available blood schizontocides include 4-aminoquinolines (chloroquine, amodiaquine), aminoalcohols (quinine, mefloquine, lumefantrine and halofantrine), and dihydrofolate reductase inhibitors (proguanil, pyrimethamine). Quinine and mefloquine share the same quinoline ring as the 4 aminoquinolines (Basco and Lebras, 1993a).

Because of the crossresistance between chloroquine and amodiaquine in highly chioroquine resistant areas in Asia and the possibility of hematologic and hepatic toxicity of amodiaquine when it is used for chemoprophylaxis, the World Health Organization (WHO) no longer recommends the use of amodiaquine for malaria treatment or prophylaxis (WHO, 1990) However, clinical studies conducted in Africa demonstrate the high efficacy of amodiaquine and

lack of serious toxic effects (Nevil *et al.,* 1994). The *in vitro* data also suggests the high activity of amodiaquine against most chloroquine resistant isolates and tend to support the clinical efficacy of amodiaquine in central and West Africa (Basco and Labras, 1993a).

Cross-resistance between chloroquine and amodiaquine suggested by these *in vitro* studies has so far not been observed in the clinical practice in Yaounde.(Basco and Ringwald, 2003) instead the *in vitro* studies have suggested the presence of a considerable number of quinine resistant isolates (up to 25%) in southern Cameroon (Myint and Shwe, 1987). An *in vitro* positive correlation of the response between two antimalarial drugs may parallel clinical cross-resistance in an endemic region where resistance to one of the drugs is firmly established, as in the case of chloroquine and quinine or chloroquine and monodesethylamodiaquine in Southeast Asia (Watt *et al.,* 1992). However, depending on pharmacokinetic factors as well as the level of resistance, an *in vitro* positive correlation may not necessarily be predictive of a clinical cross resistance.

Studies have further suggested that halofantrine may not be effective in mefloquine-resistant areas (Rooney and Thimasarn, 1991). Therefore, to resolve whether cross-resistance between aminoalcohols and artemisinin exists, a clinical study is probably needed in mefloquine-resistant areas (Caligaris *et al.,* 1992). However, another study suggested a close relationship between the *in vitro* response to mefloquine, halofantrine, and artemisinin derivatives. This might have been attributed to three possible common features between the two aminoalcohols, mefloquine and halofantrine, and artemisinin. Findings indicate that either heme itself or heme polymerase within the food vacuole may be the specific target of the 4-aminoquinolines and aminoalcohols (Slater and Cerami, 1992). Artemisinin also reacts with hemin before generating free radicals within the food vacuole of the parasite (Meshnick *et al.,* 1991). Secondly, another possible target for both mefloquine and artemisinin is membrane phospholipids (Chevli and Fitch, 1982). The

interaction between the erythrocyte membrane and artemisinin may involve the formation of a covalent linkage with the peroxide bridge, (Edwards *et al*., 1992) leading to a disruption of the membrane structure. Third, artemisinin has a tetracyclic ring, while mefloquine and halofantrine are tricyclic compounds (Pan *et al.,* 1989).

The interatomic distance between the oxygen atom in the carbinol group and the aliphatic nitrogen atom of aminoalcohols is approximately 3A (Karle *et al.,* 1992). This structural feature is probably required for intramolecular or intermolecular hydrogen bonding with the drugs' target, forming a tetracyclic structure at the level of the target molecule. Further biochemical studies of these antimalarials may reveal other common structural requirements for their specific action against malaria parasites. The limited therapeutic options for drug-resistant *Plasmodium falciparum* and the development of resistance to drugs other than chloroquine necessitate search for new drugs (Basco and Lebras, 1993a).

In Kenya, malaria continues to cause significant morbidity and mortality, and is often a location where drug resistance occurs in Africa. Both *Plasmodium vivax* and *Plasmodium falciparum* have developed resistance to numerous antimalarial drugs, which has undermined the available options for prophylaxis and treatment (Mordmuller and Kremsner, 2006). Drug resistance is the greatest challenge in the fight against malaria and is one of the major obstacles for effective malaria control in Africa.

Studies have revealed that drug resistance is associated with genetic mutations in the targeted genes. CQ resistance is linked to the major point mutation at codon 760 CQ resistance transporter genes (Wellems and Plowe, 2001). While antifoliate resistance is determined by point mutation in the dihydrofolate reductase and dihydrofolate synthasase genes (Ouellette, 2001). Multidrug resistance gene (pfmdr 1) has been shown to cause resistance to both CQ (Hayton and

Su, 2004) and lumefantrine (Sisowath *et al.,* 2005). Pyrimethamine (Amin *et al.,* 2007) resistance is conferred by the key mutation at codon 108 in the pfdhfr gene while additional mutation at position 51 and 59 increases resistance levels (Peterson *et al*., 1990).

Unfortunately, the incidence and specificity of malaria drug resistance is not homogeneous throughout the world. Different geographical locations within the same country can yield malaria parasites with various degrees of sensitivity to commercially available drugs (Mbaisi *et al.,* 2004). This warrants continued *in vitro* drug IC_{50} monitoring of field isolates. Assessment of both existing drugs and new antimalarials, alone or in combination, requires reliable methods for high-throughput testing. These findings support the need for valid surveillance efforts to predict and determine the level of malaria drug resistance.

Several strategies to monitor anti- malarial drug resistance have been proposed. Surveillance is important for the early detection of antimalarial drugs with decreased efficacy and the consequent updating of drug policies (Noedl *et al.,* 2003). The global scope of malaria and the spread of drug-resistance make the need for improved therapy undeniable (Guerin *et al*., 2002). For decades, antimalarial drug effects have been measured *in vitro* by quantifying parasite uptake of radioactive substrates as a measure of growth and viability in the presence of the test drug (Elabbadi *et al.,* 1992). While these methods are accurate and reliable, they rely on relatively expensive materials and equipment and involving procedures that become challenging with high number of samples to be tested.

2.4. Drug assaying techniques

Malaria remains one of the leading causes of childhood morbidity and mortality worldwide, especially in Africa (Breman *et al.,* 2004). For several decades, efforts to control malaria have been severely compromised by the emergence of resistance to inexpensive and widely used

drugs, such as chloroquine and sulfadoxine- pyrimethamine (Bjorkman and Bhattarai, 2005). As long as chemotherapy remains a key factor in the fight against malaria, constant monitoring of parasite susceptibility to antimalarial drugs is of the utmost importance for the development of therapeutic guidelines and policies. Simple and reliable methods for the assessment of antimalarial drug resistance, particularly under field conditions, have therefore become more important than ever before.

There are essentially two different approaches to the assessment of drug resistance in malaria parasites: *in vivo* and *in vitro* assays (Noedl *et al.*, 2003). The most traditional approach is the assessment of therapeutic responses (*in vivo* test). This 'gold standard' method has enabled determination of the thresholds of treatment failure that are crucial for adjusting antimalarial drug policies (WHO, 2003). However, the risk of loss of patients during long follow up periods and the logistical demands of clinical response studies in endemic areas have led to the development of laboratory strategies for studying antimalarial drug resistance. Laboratory strategies include *in vitro* drug sensitivity tests and evaluation of molecular markers associated with drug resistance (Bickii *et al.*, 1998).

2.5 *In vivo* versus *in vitro* drug assay techniques

There are two general methods to assess drug efficacy in the field: *in vivo* and *in vitro* tests. In the past, both WHO standard tests were largely applied in the field but accumulated experience has shown that neither of these tests has been adopted widely for making decisions on drug policies (Bruce *et al.*, 1984). The simplified *in vivo* test is performed by a regular measurement of body temperature and microscopic examination of blood films (OPS-OMS 1998) while *in vitro* assays are based on culturing *Plasmodium falciparum* isolates in the presence of a range of concentrations of an antimalarial drug for one life cycle or part of a life cycle.

Drug efficacy is assessed by counting the number of parasites developing into schizonts (WHO 2001) or by measuring the quantity of radiolabelled hypoxanthine, (Chulay *et al.*, 1983) a DNA precursor, incorporated into the parasites (isotopic microtest) (Payne and Werndorfer 1989) or by using non-radioisotope Microtests using fluorescent DNA dyes such as SYBR Green I (Smilkstein *et al.*, 2004)) Resistance is deduced when the parasite growth is not inhibited below a threshold concentration.

In vivo studies have been considered the gold standard for drug resistance (OPS-OMS 1998) and preferred for evaluation of sensitivity of *Plasmodium falciparum* to antimalarials simply because they take into account host, parasite and drug interactions, therefore acting as the reference method to detect drug resistance (WHO 2003). It is considered an accurate and valid measure of therapeutic efficacy therefore is the most reliable means for detecting drug resistance. Compared with *in vitro* assays, the *in vivo* test of resistance may be conducted in remote areas by qualified personnel with minimal training. It also permits working directly with malaria-infected patients, deriving clinical data, monitoring clinical response over a short time frame, and modifying treatment in case of a therapeutic failure (Noedl, 2003).

However, *in vivo* studies are costly and logistically complex in countries that have a low-moderate intensity of transmission with limited access to malaria endemic areas. Additionally, the tests are affected by variables that might be out of control such as absorption deficit due to the diet (Tulpule and Krishanaswamy, 1982; 1983; Romero *et al*., 1993) or other drugs (Mahmoud *et al*., 1994), increased degradation of the drug into inactive metabolites and presence of intestinal parasite infections (McElnay *et al.*, 1982).

The limitations of clinical studies for monitoring antimalarial drug efficacy highlight the need for alternative surveillance methods. Although *in vitro* methods do not replace *in vivo* methods,

they allow for the detection of intrinsic variations in parasites and help identify when and where to conduct efficacy surveys (Ruebush *et al.,* 2003). As a drug resistance surveillance tool, one of the advantages of *in vitro* methodologies is the ability to measure *P. falciparum* susceptibility to several antimalarial drugs simultaneously in the field (Bacon *et al.*, 2007). This allows for the early detection of changes in the intrinsic parasite response to antimalarial drugs, helping to prioritize, in terms of timing and location, efforts to further evaluate drug efficacy through *in vivo* surveys (Sibley *et al.,* 2008).

It has been argued that the results of *in vitro* tests of resistance do not always coincide with those of *in vivo* tests and may thus be irrelevant for clinical studies (WHO 1996). Part of the problem is associated with the type of *in vitro* assay as well as insufficient data on *in vitro* assays performed in parallel with *in vivo* tests in individual patients. For example, a comparison of *in vivo* and *in vitro* tests of resistance has not been extensively investigated for chloroquine because this drug had lost its efficacy in many endemic areas before *in vitro* culture techniques were developed and its use is no longer recommended to treat *P.falciparum* infections in these areas (Bjorkman and willcox, 1986). However, several previous studies have been conducted to assess the correspondence between *in vivo* and *in vitro* responses to antifolate drugs (Peterson *et al.,* 1990).

The results of these studies are not comparable because of differences in the *in vitro* techniques used and in the interpretation of results and are not significant due to the small sample size of field isolates. In addition, antifolate drugs are administered in combination *in vivo*, and the *in vitro* activity of the two drugs in fixed concentrations may not accurately reflect the *in vivo* conditions. Most other previous studies have performed *in vivo* and *in vitro* evaluation of drug efficacy separately (Warsame *et al.,* 1991). The real implications of these studies, especially those that were

based exclusively on *in vitro* assays or *in vivo* tests on asymptomatic patients, are not clear clinically or epidemiologically (Brasseur *et al.*, 1987).

Host factors that play an important role in therapeutic failure include variability in pharmacodynamics and pharmacokinetics and the level of acquired immunity (Basco and LeBras 1993b). There may be other factors that contribute to or delay parasite and fever clearance, such as intrinsic virulence of parasite strains, host genetic factors unrelated to immunity, concomitant diseases that were undiagnosed at the time of patient enrolment, and social behaviour of the host such as concomitant self-medication with other classical antimalarial drugs or traditional herbal medicines.

These considerations show that, unless such factors are excluded, a case of therapeutic failure cannot be attributed to *in vivo* drug resistance with certitude. These limitations may diminish the precision of the *in vivo* test and need to be taken into consideration in assessing our findings since they lead to decreased measures of validity of the *in vitro* test (Schapira *et al.*, 1986). *In vitro* assays may be a more objective method to detect drug resistance since *in vitro* tests eliminate these host factors that interfere with the clear interpretation of results, including reinfections, immunity, pharamacodynamics, and pharmacokinetics (Basco and LeBras 1993b).

In vitro assays are complementary to *in vivo* tests and their results are theoretically more directly associated with drug resistance (Wernsdorfer, 1994). However, most specialized laboratories that conduct *in vitro* assays as a routine procedure are located far from clinical study sites and require a high level of training and technical capability, transport of blood samples from the field and sophisticated equipment to perform isotopic assays (Trager and Jensen, 1976). As a result, *in vitro* assays have been used to describe the epidemiology of drug resistance independently of clinical studies and to screen new compounds (Desjardins *et al.*, 1979). Although these two applications of

in vitro assays have provided important information, the results of the study (Ringwald and Basco, 1999) suggest the usefulness of *in vitro* assays as a complementary diagnostic tool for drug resistance but do not suggest that the *in vitro* test can replace the *in vivo* test in the field.

Another major problem with the *in vitro* test is the selection of threshold values to classify results in terms of sensitivity or resistance. Use of the therapeutic plasma level as the threshold value is theoretically plausible but disregards the technical constraints of the *in vitro* culture method (Trager and Jensen, 1976). The optimal conditions for *in vitro* culture are markedly different from those for *in vivo* conditions, and include the composition of culture medium, haematocrit (1–2.5% for *in vitro*, 35–45% for *in vivo* tests) and proportion of serum (10% for *in vitro* culture 55–65% for *in vivo*) (Greary *et al.*, 1983). Thus therapeutic plasma levels may not be appropriate for *in vitro* parasite growth conditions. Comparison of different clones or laboratory adapted strains of parasites and determination of the limiting drug concentration that produces a response in these reference strains has also been used to estimate the threshold value (Ringwald and Basco, 1999). Even if the drug response of the original isolate from which a clone or strain was derived is known, adaptation of parasites to *in vitro* conditions alters the original phenotype and may not reflect the characteristics of the original isolate (LeBras *et al.*, 1983).

In addition, threshold values determined using this method may not be clear cut for some isolates obtained in the field because of the presence of mixed populations of parasites with different phenotypes. Thus, although a clone or strain *Plasmodium falciparum* of with well-defined phenotype and genotype may be useful in laboratory experiments, various *in vivo* factors preclude direct comparison between *in vitro* and *in vivo* conditions; consequently, any threshold value for differentiating sensitive and resistant isolates may largely remain arbitrary unless large scale trials are conducted under various epidemiological conditions to define

simultaneously *in vitro* drug sensitivity pattern, genotype, pharmacokinetic parameters and immune response (Wernsdofer, 1994).

Both *in vitro* and *in vivo* tests of resistance have their limitations and in any case do not measure the same biological phenomena. Results (Ringwald and Basco, 1999) show that the *in vitro* test of resistance is a complementary tool that is moderately concordant with the simplified *in vivo* test. The use of *in vitro* tests should be limited to research purposes to provide baseline data on drug response and monitor cross-resistance patterns (Warsame *et al.*, 1991). The *in vitro* test cannot replace the *in vivo* test for therapeutic efficacy and should not play any role in guiding antimalarial drug policy. Although it may be difficult to define exactly the criteria for *in vivo* antimalarials resistance and to fulfil them, especially in the field, a standardized *in vivo* test based on all available clinical and epidemiological information is still the best available means for defining drug resistance within a given epidemiological context (Ringwald and Basco, 1999).

2.6. *In vitro* drug assay techniques

Several in vitro drug sensitivity assays, based on culturing isolates in the presence of a range of antimalarial drug concentrations are available (Noedl *et al.*, 2003), and include the WHO Microtest (Bruce-Chwatt *et al.*, 1984), the radioisotope [^{3}H] hypoxanthine incorporation (Desjardins *et al.*, 1979), or *Plasmodium* lactate dehydrogenase immune detection (Kaddouri *et al*., 2006) have for a while been the techniques of choice. Lately, histidine-rich protein II assay (Noedl *et al*., 2005), fluorogenic DNA dye based assays 4, 6-diamidino-2-phenylindole (DAPI) (Banieck *et al* 2007), Picogreen (Kosaisavee *et al.*, 2006), and SYBR Green I (Smilkstein *et al.*, 2004) have been used in quantifying effects of drugs.

Each of these commonly used assays has unique advantages as well as a number of known drawbacks. Most of these traditional *in vitro* assays, such as the isotopic assay and the World

Health Organization (WHO) schizont maturation assay, have been in use for more than 20 years (Rieckmann *et al.,* 1978). However, these tests have a number of weaknesses mentioned below that limit their usefulness particularly in the field (Noedl *et al.,* 2003).

The WHO Microtest uses microscopic evaluation to enumerate the quantity of parasites following growth in the presence of antimalarials (De Monbrison *et al.,* 2003). Although this method is inexpensive, it is highly labor-intensive and subjective due to variations in the expertise of the microscopists responsible for reading the slides. This technique has never lost popularity because it is easily affordable and doesn't require expensive reagents and equipment as compared to other methods mentioned above. Additionally, it is appropriate for performing shorter *in vitro* assays since it requires relatively shorter duration (24hrs) for cultures to grow thus becomes an ideal *in vitro* technique for clinical studies. This method has also been successfully adapted for the determination of drug response of field isolates and for monitoring the changing trends of the epidemiology of drug resistant malaria parasites (Childs *et al.,* 1988).

The most widely applied method for the determination of IC_{50} values is the isotopic assay. However, this method is time-consuming and the need for the disposal of hazardous radioactive materials has limited its widespread use, especially in poor countries. Furthermore, reagents are difficult to obtain due to import and safety requirements (Desjardins *et al.,* 1979).

Two enzyme-linked immunosorbent assay (ELISA)-based methods that use monoclonal antibodies to *Plasmodium* spp. or plasmodial lactate dehydrogenase (pLDH) (Makler *et al.,* 1993) and histidine-rich protein II (HRPII) (Noedl *et al.,* 2005) gained popularity in the past few years due to their ease of use. However, the test kits can be difficult to obtain, and the performance of these multiple-step assays is time-consuming. Currently, the pLDH monoclonal antibodies are not available for purchase. In areas where genetic diversity is high, the use of

monoclonal antibodies can be hindered by a decrease in binding affinities due to genetic variation within the protein (Happi *et al.,* 2004), leading to a decrease in sensitivity and low IC_{50} values.

A microfluorimetric method using SYBR Green I for assessing susceptibility of parasites to antiplasmodial compounds was recently reported (Bennett *et al.,* 2004). This fluorescence-based method has, in particular, the advantages of being a simple, one-step procedure that is cost-effective, and the dye is readily available from multiple sources worldwide (Bacon *et al.,* 2007).

2.7. The modern *in vitro* technique; SYBR Green 1 assay

Since the 1978, measuring *in vitro* drug 50% inhibitory concentrations (IC_{50}) against field isolates (Trager and Jensen, 1978), coupled with molecular analysis, and has been useful for tracking development of *in vivo* drug resistance, from Southeast Asia to Sub-Saharan Africa (Webster *et al.,* 1985). Recently, non-radioisotope microtests using fluorescent DNA dyes such as SYBR Green I, which reliably depict *in vitro* parasite replication, have gained popularity by reducing some hurdles associated with *in vitro Plasmodium falciparum* drug sensitivity assays.

The SYBR Green I assays are considered convenient, relatively rapid, reproducible, and less costly than radioisotope assays (Smilkstein *et al.,* 2004). It can also be used in high parasite densities (Bacon *et al.,* 2007). The assay is exceptionally fast since one reagent is added to the plate after parasite growth and requires no washing and filtering steps (Bennett *et al.,* 2004). SYBR Green 1 is among the most sensitive stains available for detecting double stranded DNA (Rengarajan *et al.,* 2002). It also detects single stranded DNA and RNA at lower sensitivities (Vitzthum *et al.,* 1991). The dye is mostly preferred in gel electrophoresis to ethidium bromide because it is 50-100 times more sensitive to nucleic acid. Its sensitivity results from a remarkable

affinity for DNA and a marked fluorescence enhancement caused by the SYBR Green 1 nucleic acid intercalation (Skeidsvall and Veland, 1995).

Furthermore, SYBR Green I has replaced ethidium bromide in molecular biology because of its features. It is an asymmetrical dye binding to a double stranded DNA preferring G and C base pairs (Bennet *et al*., 2004). When intercalated to DNA it's highly fluorescent, absorbing light at a wavelength between 390 and 505nm, with a maximum excitation peak at 497nm and minor peaks at 290 and 380nm respectively (07567, 2001). These features suggest SYBR Green I drug sensitivity assays could be deployed to field laboratories, proximal to *P. falciparum* collection site. It is increasingly accepted as an alternate to ^{3}H-hypoxanthine uptake assays (CoEM *et al.*, 2009). Although fast and inexpensive the dye is not specific to malaria DNA and thus can bind to any double stranded DNA (Vossen *et al.*, 2010), resulting to high background readings. As a result causing contamination and reduces the sensitivity of the dye.

2.8 Culturing of *Plasmodium falciparum*

Culturing of *Plasmodium falciparum* is also termed as cell culture which is one of major techniques in the life sciences. It is the general term used for the removal of cells, tissues, microorganisms, or organs from an animal or plant and their subsequent placement into an artificial environment conducive to their survival and/or proliferation (Bottenstein *et al.*, 1979). Basic environmental requirements for cells to grow optimally are: controlled temperature, substrate for cell attachment, and appropriate growth medium and incubator that maintains correct pH and osmolality (Freshney, 2005).

A typical culture medium is composed of a complement of amino acids, vitamins, inorganic salts, glucose, and serum as a source of growth factors, hormones, and attachment factors. In addition to nutrients, the medium also helps maintain pH and osmolality (Darfler, 2000). Animal

cells can be cultured either using a completely natural medium or an artificial/synthetic medium along with some natural products (Mendelson, 2001).

Natural media consist solely of naturally occurring biological fluids. Natural media are very useful and convenient for a wide range of animal cell culture. The major disadvantage of natural media is its poor reproducibility due to lack of knowledge of the exact composition of these natural media. Artificial or synthetic media are prepared by adding nutrients (both organic and inorganic), vitamins, salts, O_2 and CO_2 gas phases, serum proteins, carbohydrates, cofactors (Vandemark and Smith, 1994). Different artificial media have been devised to serve one or more of the following purposes: 1) immediate survival (a balanced salt solution, with specific pH and osmotic pressure); 2) prolonged survival (a balanced salt solution supplemented with various formulations of organic compounds and/or serum); 3) indefinite growth; 4) specialized functions.

Because of the difficulty in adapting parasites from patient bloods to *in vitro* culture and the labor intensive procedures needed to maintain parasite cultures, drug tests are often performed using parasites isolated freshly from patient blood (WHO, 2005). Many factors can affect drug test results. Because most tests evaluate parasite growth by measuring the amount of parasite proteins/DNA in a sample after drug treatment, differences in parasite growth rate or developmental state may affect the test results.

In vitro drug tests are usually performed using either culture adapted parasites or parasites in blood samples freshly isolated from patients. Although selection of a dominant clone during culture adaptation is a potential concern when using culture adapted parasites, (Jefari *et al.,* 2004) drug tests using parasites collected directly from patients also have various potential problems First, it is known that fresh isolates may not grow well initially in *in vitro* culture, and some parasites eventually die during culture because of problems in

surviving *in vitro* culture conditions, not because of drug effect. Second, factors in patient blood, such as medicines taken by the patient before the tests and/or host antibodies against a particular "strain" can also contribute to inaccuracy in drug tests. Third, patient blood samples usually have very different parasitemia, and most drug assays are sensitive to variation in parasitemia. Fourth, limited supplies of patient blood will not allow repeated tests at different times, because blood is usually drawn once and patients are treated with antimalarial agents thereafter (Liu *et al.,* 2008).

The basic principles of *in vitro* drug sensitivity assay, which is an application of *in vitro* culture of malaria parasites, have not changed since Trager and Jensen discovered the suitable experimental conditions for continuous *in vitro* culture of *Plasmodium falciparum*. These include RPMI 1640 culture medium buffered with 25mM N-(2-hydroxyethyl) piperazine-N -(2-ethanesulfonic acid [HEPES]) and 25 mM NaHCO3 and supplemented with 10% human serum, a thin layer of infected erythrocytes, and incubation in an atmosphere of low oxygen at 37°C (Trager and Jensen 1976). Of these requirements, the need for human serum is a major limitation that hinders a wide application of standardized *in vitro* drug sensitivity assays in endemic countries (Basco, 2003).

Repeated tests under similar conditions are critical for malaria drug assays, because variations from test to test are frequently observed. Regardless, *in vitro* assay surpass the *in vivo* assay as discussed earlier, therefore calls for identification of a proper medium to support viability of plasmodia from the field to the laboratory, and another to provide maximum growth when cultivated in the presence of antimalarials. A transport medium enables the transportation of microbes to the lab for culturing while growth medium or culture medium is a liquid or gel designed to support the growth of microorganisms, or cells. Cell culture media generally

comprise an appropriate source of energy and compounds which regulate the cell cycle (Karmiol, 2000).

2.9 Proposed transport medium for *Plasmodium falciparum*

A good transport medium provides a "physiological" or balanced salt solution. The functions of this salt solution in the medium are to maintain proper pH, maintain ideal osmotic pressure, and provide a source of energy. This environment seeks to reduce the shock experienced, which can lead to death due to the abrupt change of environment from the human body to the outside environment (Freshney, 2005). This enables survival of microorganisms during transportation to the laboratory for further cultivation. The growth of animal cells in a nutritionally complete tissue culture medium is usually optimal when the medium is buffered at a pH in the range of 7.2-7.4. To function most effectively, the pKa of the chosen buffer should be as close as possible to the required pH (Eugui and Almquist, 1990). The components of this medium are trisodium citrate, Hepes, Sodium bicarbonate and RPMI1640.

2.9.1 Trisodium citrate

Sodium citrate is a white crystalline powder with a chemical formula $Na_3C_6H_5O_7$. It's soluble in water at 20^oc and insoluble in ethanol (Vandemark and Smith, 1964). Successfully used as an anticoagulant in blood transfusions, thus being used today in blood collection tubes and for the preservation of blood in blood banks (Shi, 1993). The citrate ion chelates calcium ions in the blood by forming calcium citrate complexes, disrupting the blood clotting mechanism (Echezarreta, 1997). As a conjugate base of a weak acid, citrate can perform as a buffering agent or acidity regulator, resisting changes in pH, it can buffer solutions in approximately the pH 3.0 to 6.3 range (Voopik *et al.*, 2003).

2.9.2 Hepes

Hepes is a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid with a dissociation constant of ($pK_{a)}$ of 7.5 at 25 °c and is useful at pH range of 6.8 to 8.2 (Shipman, 1969). It's a zwitterionic organic chemical buffering agent; one of the twenty Good's buffers. HEPES is widely used in cell culture, largely because its Co_2 independent unlike bicarbonate buffers (Zigler *et al.,* 1985), thus is better at maintaining physiological pH despite changes in carbon dioxide concentration (produced by cellular respiration) and also when cell cultures require extended periods of manipulation outside of a CO2 incubator (Uszynski, 2007). The dissociation of water decreases with falling temperature, but the dissociation constants (pKa) of many other buffers do not change much with temperature. HEPES is like water in that its dissociation decreases as the temperature decreases (Freshney, 1992). This makes HEPES a more effective buffering agent for maintaining enzyme structure and function at low temperatures (Baicu and Taylor, 2002) Lepe-Zuniga and others reported a phototoxicity of HEPES when exposed to ambient light by the production of hydrogen peroxide, (Lepe-Zuniga *et al.* 1987) which is not a problem in bicarbonate-based cell culture buffers. It is therefore strongly advised to keep HEPES containing solutions in darkness as much as possible (Nagle, 1999).

2.9.3 Sodium bicarbonate

Sodium bicarbonate is important to be maintained in the medium since sufficient bicarbonate is important for nutritional purposes. Bicarbonate also has many metabolic functions as well as buffering capacities (Freshney, 1994). However, this buffer has two important disadvantages. First, the pKa of sodium bicarbonate is 6.3 at 37°C which results in suboptimal buffering throughout the physiological pH range (Li and Schlessinger, 1991). Secondly, since its Co_2 dependent, when carbon dioxide is released in the atmosphere there is a resulting increase in alkalinity, and the number of hydroxyl ions produced increases according to the amount of

sodium bicarbonate added to the medium (Davis, 1994). Sodium is one of the inorganic salts in the media which helps to retain the osmotic balance and also helps in regulating membrane (Cinatl, 1969).

2.9.4. RPMI 1640

RPMI-1640 is a powder which has L-glutamine as an essential amino acid, (lane *et al.,* 1987). It is a general purpose media with a broad range of applications for mammalian cells, especially hematopoietic cells. RPMI-1640 was developed at Roswell Park Memorial Institute (RPMI) in Buffalo, New York (Pasiek *et al.,* 1959).

Amino acids are the building blocks of proteins, and thus are obligatory ingredients of all known cell culture media (Stoll *et al*., 1996). Essential amino acids must be included in the culture media as cells cannot synthesize these by themselves. They are required for the proliferation of cells and their concentration determines the maximum achievable cell density (Evavold and Allen, 1991). L-glutamine provides nitrogen for NAD, NADPH and nucleotides and serves as a secondary energy source for metabolism (Romeo, 1991).

2.10 EDTA anticoagulant

Anticoagulants are used to prevent clot formation both *in vitro* and *in vivo*. In the specific field of *in vitro* diagnostics, anticoagulants are commonly added to collection tubes either to maintain blood in the fluid state for hematological testing or to obtain suitable plasma for coagulation and clinical chemistry analyses. Unfortunately, no universal anticoagulant that could be used for evaluation of several laboratory parameters in a sample from a single test tube is available so far (Narayanan, 1993).

Ethylenediamine tetraacetic acid (EDTA) is a polyprotic acid containing four carboxylic acid groups and two amine groups with lone pair electrons that chelate calcium and several other metal ions (Goossens *et al.,* 1991). Calcium is necessary for a wide range of enzyme reactions of the coagulation cascade and its removal irreversibly prevents blood clotting within the collection tube. Historically, EDTA has been recommended as the anticoagulant of choice for hematological testing because it allows the best preservation of cellular components and morphology of blood cells (Reardon *et al.,* 1991).

Heparin is used for clinical chemistry, sodium citrate for coagulation tests, and (EDTA) for hemocytometry (Petrotta *et al.,* 1998). The main property of EDTA, is the ability to chelate or complex metal ions in 1:1 metal-EDTA complexes (Goossens *et al.,* 1991). Owing to its strong complexation with metal ions that are cofactors for enzymes, EDTA is widely used as a sequestering agent to prevent some enzyme reactions from occurring.

When blood is collected with no additives within an appropriate container (blood tube), it clots fairly quickly. As calcium ions are necessary for this process, the specific association between the carboxylic groups of EDTA and calcium is a reliable solution to prevent clotting, stabilizing whole blood in a fluid form, as required for some laboratory analyses. EDTA has been chosen for hematological tests when aniline derived dyes were proposed for preparing blood smears from peripheral venous blood (FESCC, 2006).

EDTA allows optimal dying with Giemsa stain. Heparin, conversely, triggers platelet (PLT) activation, is more expensive and affects the staining properties, producing a

reddish coloration. Citrate is used as an anticoagulant primarily for coagulation studies (Petrotta *et al.*, 1998).

Moreover, EDTA showed optimal extended stabilization of blood cells and particles. Owing to these properties, EDTA became the anticoagulant of choice for hematological testing. An additional advantage of EDTA is that it protects labile molecules; therefore, it may be used to preserve labile analytes, possibly in association with antiproteolytic substances (Reardon *et al.,* 1991).

The stability of hematological parameters in blood stored in EDTA is excellent: 48 hrs for hemoglobin (Hb) and 24 hrs for red blood cells (RBC) parameters using impedance technologies (Kouri *et al.,* 2005). Leukocytes are stable for 24 h when stored at 4^0C; the cell differential is also stable in refrigerated samples (Lippi *et al.,* 2005). Reticulocytes tend to mature and transform to RBCs in whole blood, but the stability is high (72 h) if EDTA anticoagulated blood is stored at 4^0C (Cavill *et al.,* 1996).

It is therefore acceptable to use EDTA as the anticoagulant of choice if delayed blood processing is anticipated. It also has the advantages of a lack of inhibitory action on *Taq* DNA polymerase, compared with heparin, and negligible volume, compared with acid citrate dextrose (Basco, 2004).

2.11. Need for Antimalarial Sensitivity study

Malaria kills 610,000 – 971000 people annually with majority of the deaths occurring in sub-Saharan Africa (WHO, 2012). Efforts on preventive measures and availability of effective therapies have not been successful in controlling malaria. Uses of insecticides impregnated and treated nets, indoor residual spraying, environmental modifications, have faced setbacks in the recent years (Vulule *et al.,* 1999; Nguessan *et al.,* 2007; Yewhalaw *et al.,* 2010). Compexity of

the *Plasmodium* parasite and its lifecycle (Gardner *et al.,* 2002) poor understanding of the interaction between the parasite and the immune system (Langhorne *et al.,* 2008) and extensive antigenic variation (Scherf *et al.,* 2008) have hampered vaccine development efforts.

Antimalarial drugs, in combination with mosquito control programs, have historically played a key role in controlling malaria in endemic areas, resulting in significant reduction of the geographic range of malarial disease worldwide (Florens *et al.,* 2002). However, the continued emergence and spread of multi drug-resistant strains of *Plasmodium falciparum* are arguably the most pressing problems on the area of the infectious disease today (Bennett *et al.,* 2004) hence turning back the clock on control efforts (WHO, 2010).

Inappropriate supply and use of antimalarials emerges the major contributory factor. Self-medication with antimalarials is a global problem. It has been reported in several parts of the world; Yemen (Abdo-Rabbo 2003), Sudan (Awad *et al.,* 2005), Togo (Deming *et al*., 1989), and Ethiopia (Deressa *et al*., 2003). Because of the lack of access to health care facilities, many residents of sub-Saharan Africa receive their initial treatment for febrile illnesses at home using herbal medicines, oral antipyretics, or antimalarial drugs purchased without prescription in local shops (Glik *et al.,* 1999; Raynal, 2005; Kaseje *et al.,* 2007). Even when malaria is suspected and antimalarial drugs are used, patients rarely take an adequate course of treatment (Dabis et al.,1999; Deming *et al.,* 2004) and many only visit a health centre or hospital after the illness has failed to respond to several days of self-treatment (Sauerborn *et al.,* 2009).

In Kenya, Mtito Andei in Eastern Kenya has reported 38% of preschool children with majority of self medication facilitated by their mothers. Antimalarials was found to be the leading drug for self-medication (Ruebush *et al*., 1995) among school going children in rural western Kenya, with chloroquine the most prefered (67%) antimalarial (Geissler *et al.,* 2000). About 50-80% of

individuals in Bungoma obtained antimalaria from private drugs outlets without prescription (Hamel *et al.,* 2001). Studies have shown that in Kisumu, 87.6% of antimalarials are non-prescribed / self-medication, out of which 70.3% are artemisinin based combination therapies (Sammy *et al.,* 2013).

Self-medication promotes selection and use of ineffective antimalarials with diminished efficacy. This behavior contributes to increased malarial burden by providing asymptomatic relief thus delaying early diagnosis and administration of curative drug regimens (Watsierah, 2011). It also leads to administration of wrong dosage, wrong frequency and wrong duration of therapy (Buabeny *et al.*, 2007). Non recommended use of such drugs can contribute to development and intensification of drug resistance through increase in exposure of parasites to sub optimal drug levels (WHO, 2000). So it is important coming up with a more sensitive, easily available and affordable technique to monitor sensitivity profiles of the combination therapies used within the region as a way of curbing development of resistance and reducing mortality due to malaria.

CHAPTER 3.0: MATERIALS AND METHODS

3.1 Introduction

This chapter elaborates on the all the materials and the methodology which was used in collecting and analyzing data: the study site, study design, study population, sample size, Data collection instruments, data collection procedures, laboratory procedures, statistical analysis and ethical considerations.

3.2 Study Site

The study included population residing within Maseno division and visiting Chulaimbo sub district hospital. This study site was randomly selected because the study required to enroll participants from a site within 30 minutes drive from the laboratory. Longer duration of transit period would affect the study by varying widely the duration between sample collection and testing. Also, Maseno division lacks data on *in vitro* drug susceptibility to serve as reference guide for the ministry of health intervention.

Maseno Division is one of the four administrative divisions in Kisumu District of Nyanza Province, Kenya, whose headquarters is Maseno town. It has four locations namely; Northwest Kisumu, Otwenya, East Seme and West Kisumu and fifteen sublocations. It is located along Kisumu - Busia highway 20 kilometers Northwest of Kisumu, the provincial capital. The altitude of Maseno is 1,503 metres or 4,934 feet above sea level. This place is situated in Western Kenya whose malaria prevalence rate is 30% (Ministry of Public Health and sanitation 2011); its geographical coordinates are 0° 10' 0" South, 34° 36' 0" East. Maseno division has a population of 65,304, of whom 2,199 are classified as urban (Electoral commission of Kenya (E.C.K) 1999). It has a tropical climate with significant rainfall throughout the year. The average annual temperature is 20.6°C and the average annual rainfall is 1820 mm. These climatic conditions are suitable for agriculture being the people's main economic activity. Availability of rainfall, warm

temperature and the presence of vegetation due to agriculture provide a good breeding and hiding place for mosquitoes, the vectors for transmitting malarial parasites in the region, thus malaria becomes one of the common disease. Chulaimbo Sub District hospital is strategically situated in the area and is the only well equipped public hospital used by residents of this region. (Appendix 4).

3.3 Study design

This was a cross-sectional laboratory based epidemiological study with samples collected from Chulaimbo sub-county hospital.

3.4 Study Population

Patients who visited Chulaimbo Sub district hospital presenting with symptoms of malaria were eligible for the study if they met the inclusion criteria. Children below 6months were not recruited for the study because they are vulnerable in management, they are mostly under 5kgs and the drugs as per WHO regulations are administered from 5kgs and above. These individuals still have passive immunity from the mothers which can affect their response to drugs (WHO PAR Part 4 2010).

Patients testing positive for malaria upon testing by microscopy were requested to participate in the study. Consent for patients who were under eighteen years of age was signed by their guardians. Consenting/ assenting individuals were requested to provide 4mls of blood samples for the study. Results obtained from the processed pre-culture thick and thin films formed the basis for the criteria. Individuals meeting the inclusion criteria below were included in the study

3.4.2 Inclusion criteria

- Permanent resident of Maseno Division, and therefore has been born there or has stayed there for atleast the last 3 years.

- Patients with Giemsa stained thin film mono-infections of *P. falciparum*
- Patients whose parasitaemia was between 1,000 to 200,000 asexual parasites/µl of blood
- Patients who consented
- Patients who were not in antimalarial medication

3.5. Sample size determination

The sample size was determined from the target population of 65,304 (E.C.K, 1999) people living within Maseno Division. Fisher's formula (Fisher *et al.*, 1998) for sample populations exceeding 10,000 was used in sample size determination as shown below. A confidence level of 95% was assumed.

$$\mathbf{n = \frac{Z^2pq}{d^2} = \frac{1.96^2 \times 0.30 \times (1-0.30)}{0.05} = 322 \text{ participants}}$$

n= minimum sample size

Z= Standard normal deviate at the required confidence level (error 5% Z = 1.96)

P= Proportion of subjects in the sample population estimated to be infected by malaria is 30% (Ministry of Public Health and sanitation 2011)

q=1-p

d= Absolute precision expressed as a fraction of 100 (accuracy level of 5 % chosen = 0.05).

The minimum sample size was therefore 322 participants

3.6 Variables

3.6.1 Dependent variable

Sensitivity and resistance results of malarial parasites to anti-malaria drugs.

3.6.2 Independent variable

Plasmodium growth *in vitro* in the presence of the antimalarials

3.7 Data collection instruments.

This study used a laboratory note book to document raw data both in hospital and in the laboratory and checklists. Data from the hospital included demographic characteristics and parasitaemia level (Appendix 1), while in the laboratory, data for IC_{50} reference clones and field isolates were collected in tables shown in Appendix 2 and 3.

3.8. Data collection procedures

For the first objective, reference clones (W2 and 3D7) which had been stored in liquid nitrogen were successfully revived in KEMRI and brought to the lab in Maseno at 4^0C in plain vacutainer tubes with 2ml formulated transport medium and EDTA anticoagulants tubes for further assay. While the second objective involved collection of positive blood samples from hospital located in a 20 minutes' drive to the laboratory. Samples were collected in plain vacutainer tubes with 2ml formulated transport medium and EDTA anticoagulants tubes. These samples were kept at 4^0C until transported to the laboratory for sensitivity study within 6 hours. In the laboratory samples (clones and field isolates) were prepared and dosed to the 96well plates coated with antimalarials in various concentrations and incubated for 72hrs for parasites to grow. On the elapse of 72 hours plates were terminated and dried in the hood for an over night stay, the SYBR Green dye was added in KEMRI prior to being read using the tecan machine to give the Relative Flourescent Units from every well. Using the Graph pad prism 5 software IC_{50} values were determined for each sample between TM and EDTA using dose response curves. The IC_{50} of the clones and the field isolates were analysed to give results for the first objective and the second objectives respectively, while their correlation gave the results of the third objective.

3.9 Selection and recruitment of study participants

A total of 322 patients who presented at the outpatient clinic of Chulaimbo sub County Hospital with symptoms of malaria, diagnosed with *P. falciparum* parasites by a Giemsa stained thin blood smear and gave written informed consent to participate were enrolled. From each enrolled patient, 4mls of venous blood sample was drawn by a phlebotomist from the Ministry of Health so as to reduce the risk incurred. Upto 2ml of the blood sample was added to a normal 5ml EDTA vacutainer tube and balance of 2mls dropped into a vacutainer tube with 2ml transport medium (TM) and mixed by slowly tapping the tube. Both tubes were labeled with patient's name, time and date of collection. Both samples were placed inside a cool box and transported to the laboratory situated at Maseno University for sensitivity studies within 6 hours.

3.10. Experimental flow chart

A stabilizer should be able to keep *Plasmodium falciparum* viable while reaching the lab. This is one of the factors promoting their *in vitro* growth. EDTA is the most frequently used, however, in seeking for an alternative stabilizing media, this study proposed a transport medium with nutrients which was used to maintain samples before subjecting them to antimalarials. The reference clones (W2 and 3D7) which were maintained in a culture were exposed to EDTA and transport medium before being tested for susceptibilities to various antimalarials.Their IC_{50}s to various drugs were determined and compared with those in the literature so as to validate the lab assay and also to guide on the range of sensitivities of the field isolates. Once comparable, the field isolates which were exposed to the transport medium and EDTA were also tested for drug susceptibilities and their IC_{50}s determined. The obtained IC_{50}s for the reference clones and the field isolates were compared with the literature results so as to get the field profile susceptibility.Validity of the stabilizing agent was also obtained by correlating IC_{50} values of samples in EDTA and in the transport medium.

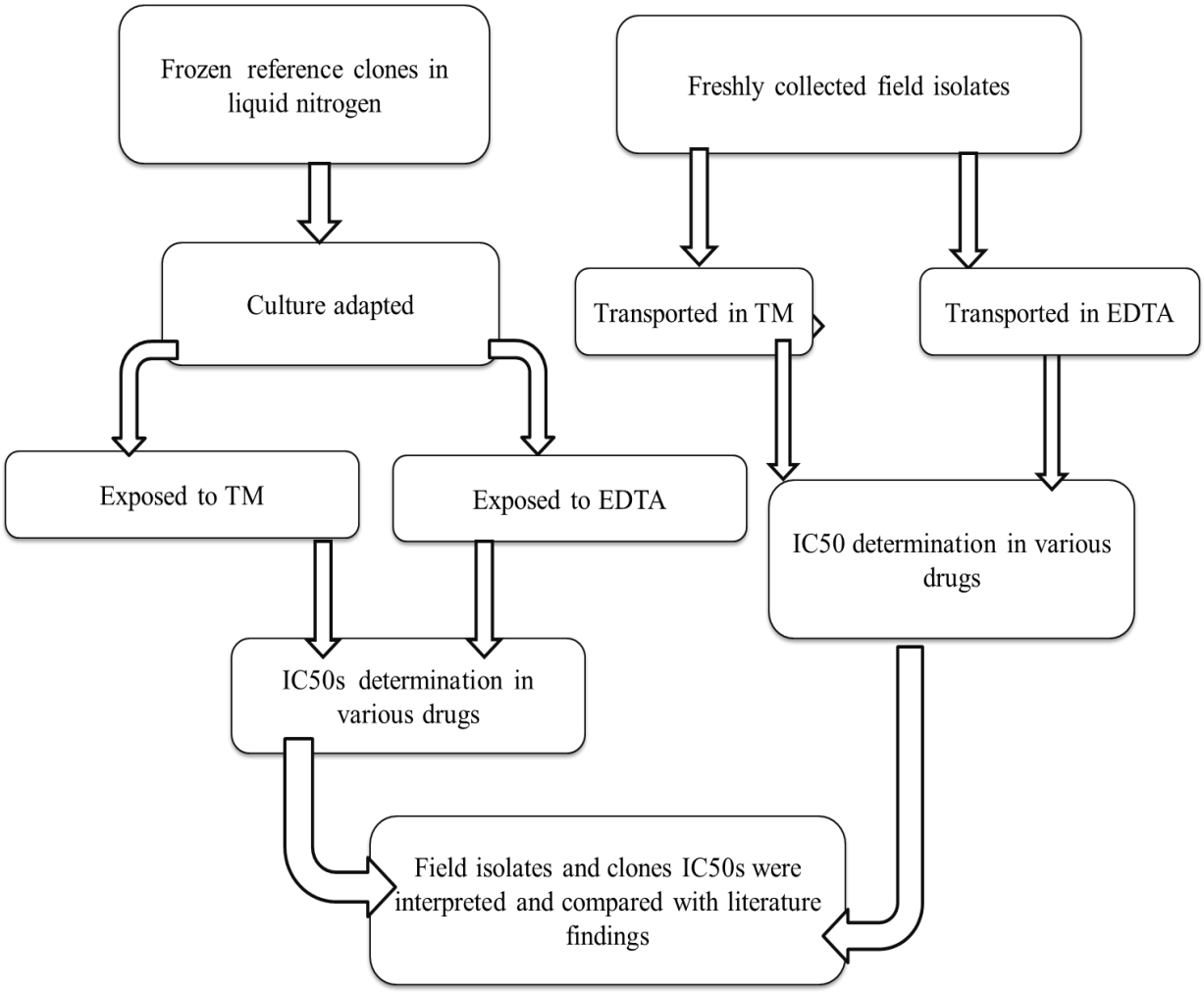

Figure 3.1. Experimental flow chart

3.11 Laboratory procedures

3.11.1. Procedures for preparation of reagents

3.11.1.1 Preparation of transport medium (TM)

The following reagents were weighed and mixed in 200ml of distilled water; 8g of trisodium citrate, 1.45g of hepes, 6.5g of sodium bicarbonate and 2.6g of RPMI 1640. The solution was stirred gently using a magnetic stirrer until all the reagents had dissolved .This was sterilized by passing through 0.2um pore filter units and kept at 4^0C for a maximum of one month.

3.11.1.2 Preparation of Phosphate buffered saline (PBS)

Phosphate buffered saline was prepared by adding 1.7g of sodium citrate and 0.7g potassium phosphate to 1litre of sterile distilled water. This was mixed thoroughly and the pH adjusted to 7.2 using concentrated Hydrochloric acid (HCL).

3.11.1.3 Preparation of Giemsa stain solution

Into a 50 ml tube, 5ml of Giemsa stain was added to 45ml of PBS and mixed. This was used within 24 hrs.

3.11.1.4 Preparation of uninfected Red Blood Cells (RBCS) and Serum

Collection of blood for uninfected RBCs and Serum was done from volunteers in malaria non endemic areas based on the inclusion and exclusion criteria below. Donor demographic information was not needed for experimemts of any other analyses under this study. Blood collected was shipped at a temperature of between +2 and $+8^0$C to the laboratory by courier, so as to reach the lab within 24 hours of blood collection for preparation.

Criteria for inclusion of volunteers

- The potential donors will be aged between 18 years and 50 years inclusive.
- The potential donors will be seen to be generally in good health as determined by the clinician following a physical examination and feeling well.
- Additionally, the clinician will ensure that the hemoglobin levels are within normal range (14 – 18g/dl for males and 12 – 16g/dl for females), and that no malaria parasites have been detected in the blood.
- Willing to give informed consent

Criteria for exclusion of volunteers

- Blood pressure that is out of range.
- Hemoglobin levels that are below acceptable levels
- Chronic illness
- Presence of any lesions or scars of needle pricks at the venipuncture sites that are indicative of addiction to narcotics and/or frequent blood donation.
- Frequent travel to malaria endemic areas – more than once a month for most months in a year
- Prior prolonged residence in a malaria endemic area – more than 3 years
- Frequent malaria episodes in the past – more than once a month for most months in a year
- Pregnancy
- Lactating mothers
- Findings that in the opinion of the investigator may result in adverse outcomes should the volunteer continue participation
- Donation of blood within the last 6 months,
- Taking antimalarial drugs or antibiotics within the last 2 weeks.

Preparation of uninfected RBCS

Red blood cells (RBC) were used to lower parasitaemia and adjust hematocrit as needed in parasite cultures and ring stage survival assays. The RBCS should be drawn from individuals from malarial non endemic areas. They should test negative for haemoglobinopathies and glucose 6 phosphate dehydrogenase (G6PD) deficiency before use. Up to 60 ml of blood group "O" was drawn from each donor. Two donors per week were required for this study.

Whole blood was collected in a 250ml pouch containing Acid Citrate Detrose (ACD). The pouch was first sprayed with 70% ethanol and left to dry in the biosafety cabinet. The pouch tube was cut with a sterile pair of scissors carefully to open. The blood was then transferred in to 50ml centrifuge tubes and stored at 4^0C for up to 3weeks. The RBCs were washed by centrifugation before using it to adjust parasitaemia in parasite cultures, This was done by transfering 25ml of whole blood and centrifuging at 2500 revolutions per minute (RPM) for 3 minutes so as to remove plasma and buffycoat .This was followed by addition of 10% CMS to a final volume of 50ml then centrifuged again to remove supernatant for at least three times. The cells were stored without mixing at 4^oC for up to 4 days while being used.

Preparation of serum (ABO)

Blood from AB and O groups were required – 400ml was drawn from each donor, into blood collection bags without anticoagulant for it to clot. For the entire study whole blood from 10 individuals were collected. The pouch was then sprayed with 70% ethanol and left to dry in a biosafety cabinet. The pouch tube was cut with a sterile pair of scissors, opened carefully and by decanting slowly not to disturb the clot, the serum was aseptically transferred into 50ml centrifuge tubes, labeled and stored at -80^0C for use indefinitely. A total of 400 ml serum was collected for the experiment.

3.11.1.5 Preparation of hypoxanthine

A volume of 40mg hypoxanthine was dissolved in 200ml of distilled water then boiled for 10 minutes. After evaporation, the volume was adjusted to 200ml by adding distilled water and cooled at room temperature. The solution was then filtered sterile with 0.2um pore filter unit and stored at 4^0C to be used indefinitely.

3.11.1.6 Preparation of 7.5% sodium bicarbonate ($NAHCO_3$)

A total of 5g sodium bicarbonate was dissolved in 100 ml distilled water and filtered sterile in 0.2um pores units then stored at room temperature to be used indefinitely.

3.11.1.7. Preparation of plain medium

To prepare 1L of plain medium, 5.2g of RPMI 1640, 5.94g of hepes and 2g of glucose were mixed using a magnetic stirrer in 1 litre of distilled water. The solution was filtered sterile in 0.2um pores of filter units and stored to be used for a maximum of one month.

3.11.1.8. Preparation of 10%complete medium with serum (CMS)

To make 1 litre of 10% CMS, a total of 118ml of ABO blood serum (59 ml of AB and 59ml of O+) which had been filter sterelized in 0.2um filtration units was added to 1 litre of plain medium solution. This was followed by the addition of 23.6ml of hypoxanthine and 37.7ml of $NAHCO_3$. The medium was used for a maximum of 2 weeks while kept at 4^oC.

3.11.1.9 Preparation of Acid Citrate Dextrose (ACD)

Upto 2.2g of trisodium citrate, 0.8g of citric acid and 2.4g dextrose were dissolved in 100ml of distilled water. This was then filtered using 0.2um pore of filter units.

3.11.1.10 Drug dilution procedure

The following stepwise procedures were used to dilute the antimalarial drugs namely Artemether, lumefantrine, Dihydroartemisinin and Piperaquine to the correct starting concentrations (Mbaisi *et al.,* 2004). Artemether, lumefantrine and Dihydroartemisinin required two steps dilutions from 5,000,000 ng/ml to 200 ng/ml while peparaquine required one step dilution from 5000000 ng/ml to 500ng/ml

To prepare the starting concentration, 2.9mg of Arthemeter was dissolved in 580ul of DMSO to make 5mg/ml (equivalent to 5000000ng/ml). This was diluted at 1:50 followed by 1:500 to give a final dilution of 100000ng/ml and 200ng/ml respectively. Same dilutions were obtained for the other drugs using the starting concentration of 5mg/ml DMSO as follows: 3.0mg lumefantrine in 600ul DMSO, 3.8mg of dihydroartemisinin in760ul DMSO. Final one step concentration of piperaquine was obtained by 5mg of the drug in 1000ul DMSO. Depending on the number of plates required to be assayed, the volume of the final concentration was adjusted accordingly without altering the concentration.

3.11.1.11 Preparation of Malaria SYBR Green 1 Fluoresence Assay (MSF) lysis buffer

To prepare 1Litre of Malaria SYBR Green 1 Fluoresence Assay (MSF) lysis buffer, 2.423g Tris base was added to 1 litre bottle of cell culture water and pH adjusted to 7.5 using concentrated hydrochloric acid. To the culture water, 10ml of EDTA solution, 80g of saponin and 0.8ml of Triton x20 were added. The solution was thoroughly mixed while avoiding the production of bubbles, after which the solution was vacuum filtered to remove particulate matter. This was stored at room temperature and could be used for a period of six months.

3.11.1.12 Preparation of SYBR Green 1stock solution

SYBR Green 1stock solution (10,000 X) was thawed at room temperature in a larminar flow hood in the dark. Aliquots of 10ul were put in to microtubes and were stored at -65^{0}C to -80^{0}C until use within 6 months.

3.11.1.13 Preparation of MSF lysis buffer containing SYBR Green1

To make MSF lysis buffer with SYBR Green sufficient to dose one plate of 96wells, 11ml of lysis buffer was mixed with 10ul of SYBR Green 1 stock solution. Thus one aliquot vial of SYBR Green 1 was thawed and the content pippeted to mix with the buffer slowly to avoid

production of bubbles. Enough solution was made fresh prior to harvest of assay depending on the number of plates to be assayed.

3.11.2 Reviving of the W2 and 3D7 clones

These were clones which were kept in liquid nitrogen and needed to be recultured for the experiment. Thawing procedure was done as follows; first the isolates were removed from liquid nitrogen and indicated in the nitrogen freezer log sheet, and then the vials were left to thaw in the incubator at 37^0C. Once the content liquefied it was transferred aseptically in to a 15ml centrifuge tube noting the volume. Slowly 1/5 volume of 12% solution Nacl was added while swirling the tube. It was allowed to stand at room temperature for 5 minutes. Nine volumes of 1.6% Nacl solution was added to it and was centrifuged at 1500rpm for 3minutes. Supernatant was aspirated before another nine volumes of 0.9% Nacl supplemented with 0.2% dextrose solution were added. This was mixed gently and centrifuged at 1500 rpm for 3min, and then supernatant was aspirated. It was resuspended in culture medium by adding 4.5ml of 10% complete medium and 0.5ml of washed RBCs for a 5ml culture. Then the culture was gassed and placed in an incubator at 37^0C. Medium was changed after every 48 hours while gassing the bottles. Parasitaemia was checked three times weekly until 3-8% was obtained (Desjardins *et al.,* 1979). They were then subjected to EDTA and formulated TM in 5ml vacutainer tubes and transported to the laboratory while kept at 4^0C in a cool box with ice packs.

3.11.3 Preparation of a Giemsa stained thick smear.

A drop of blood was placed on a slide and allowed to dry in the safety cabinet hood, giemsa stain was then flooded on the slide for 20 minutes so as to stain the parasites. Excess stain was removed by washing using running tap water gently. It was then dried before examining using oil immersion objective lens (x100).

3.11.4 Preparation of a Giemsa stained thin smear

A drop of blood was placed on the slide and spread to make a thin film using the edge of another slide; this was hit fixed and flooded with Giemsa stain for 20 minutes so as to stain the parasites. Slide was passed through running tap water gently to remove excess stain. It was then examined using oil immersion objective lens (x100).

3.11.5. Estimation of parasitaemia

Diagnosis was done by a Giemsa stained thick blood smear microscopy. Calculation of parasitaemia was done by counting the number of *P. faciparum* parasites from the Giemsa stained thin blood smear. In the thin smear slides, at least three fields of infected RBCs (with rings, trophozoites, shizonts, and gametocytes stages) against the total number of RBCs counted. The sum of the total number of RBCs infected against the total number of RBCs in the fields was obtained. This was used to calculate the % parasitaemia as shown below.

$$\frac{\text{Total no. of infected RBCs in the three fields}}{\text{Total no. of RBCs in the three fields}} \times 100$$

3.11.6 Procedure for the *in vitro* assay

On arrival of the field isolates and the revived reference clones in EDTA and TM to the laboratory, the following stepwise procedures were undertaken in processing samples for culturing.

3.11.6.1 Washing of the blood samples

Samples brought in vacutainer tubes containing EDTA and TM in a cool box were transferred aseptically to a 15ml tubes for centrifugation inside the biosafety cabinet hood to avoid contamination. The content in these tubes were labeled accordingly. Washing was done using 10%complete medium by centrifugation at a speed of 2500 revolutions per minute (rpm) for 3

minutes prior to removal of the supernatant, for three successive times. The resulting pellet was ready for loading into drugs predosed plates.

3.11.6.2 Coating of the microtitre plates with drugs

Coating of the mother plates

To make the *in vitro* antimalarial drug sensitivity test plates, sterile 96 well flat-bottom microtitre plates were dosed with decreasing concentrations of test grade antimalarial drugs (artemether, dihydroartemisinis, lumefantrine, and piperaquine,). This was done by introducing 300ml of the diluted drugs in their final concentration to each well in the first column, with every two consecutive rows (A and B, C and D, E and F, G and H) having the same drugs hence every concentration of drugs was done in duplicate, while the rest of the columns had 150ml of CMS. 150ml of the content in the first column was picked using the 200 µl fixed volume Eppendorf pipette and a disposable sterile tip and was serially diluted across the 10 columns of the plate, excluding the last 2 columns which acted as controls. This gave rise to a mother plate which was used to make 10 daughter plates for parasites culture. Several of these mother plates were made and kept at -65^0c to -80^0c for 1-2 weeks and could be freeze thawed only twice for experimental use.

Coating of the daughter plates

To make a daughter plate, 12.5ul of the content of each well in the mother plate were aseptically transferred to the wells of the sterile microtitre plates using the 200 µl fixed volume Eppendorf pipette and disposable sterile tips. Coating was done starting with the control wells (columns 12 and 11) and following an increasing order of concentrations, ending with column 1. New sterile disposable tips were fitted to the Eppendorf pipette and the next daughter plate was set up in exactly the same way, and so on until the entire scheduled daughter plates were dosed. These

plates were kept at -20^{0}c and prior to use in drug screens they were thawed in 35-37^{0}C culture incubator for 1-2hours.

Table 3.1 Layout of the microtitre plate

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
A												
B												
C												
D												
E												
F												
G												
H												

3.11.6.3 Antimalaria drugs assay procedure

Giemsa stained thin blood smears were again prepared for every sample (clone and the field isolates) to confirm the presence of parasites and to calculate the % parasitaemia. Calculated amount of washed blood was picked using a 200 µl fixed volume Eppendorf pipette and a disposable sterile tip and mixed in a trough with 10.3 ml of 10% complete medium enough to dose the 96well microtitre plate. Blood samples with > 1% parasitemia were adjusted to 1% parasitemia at 2% hematocrit, by adding prepared fresh RBCs and those with $\leq$ 1% parasitemia were used unadjusted at 2% haematocrit. About 100uL of the samples collected in the normal EDTA vacutainer tube and that in the TM tubes were transferred to the pre-coated drug plates separately. Dosing was done starting with the control wells in column 11 and 12 and following an increasing order of concentrations, ending with wells in column 1. The lids were replaced on the microtitre plates and contents labeled accordingly. The plates were shaken gently, without

lifting it from the work bench, so that the drugs would be homogeneously suspended in the assay components.

The plates were placed in tight ziplock bags with humidity provided by wet paper towels and gassed with mixed gas of 90%N, 5%Co_2 and 5%O_2. After which they were incubated in 37^0C for 72 hours to allow growth of the parasites. To facilitate reading of results, the plates were dried in the hood overnight then kept in tightly sealed ziplock bags until they were taken to KEMRI for reading. At KEMRI, the plates were rehydrated by adding100 uL of lysis buffer containing SYBR Green I (1 X the final concentration) to each well of the plate and were mixed gently. The plates were then incubated for an overnight stay at room temperature in the dark. Relative fluorescence units (RFU) per well were examined using the Tecan Genios Plus flourimetre (Tecan US, Inc., Durham, NC).

3.12. Statistical analysis

The data obtained was entered in an Excel sheet (2010 version) in a central computer with a password in the laboratory. Parasite replication inhibition was quantified and the IC_{50} for each drug calculated by an equation generating a sigmoidal concentration-response curve (variable slope), with log transformed drug concentrations on the X axis and relative fluorescent units (RFUs) on the Y axis (Graphpad Prism for Windows, version 5.0; Graphpad Software, Inc., San Diego, CA (Johnson *et al.,* 2007). Using the same software IC_{50} data were analysed by using Mann-Whitney test; a non parametric test which doesn't assume a gaussian distribution, to compare the medians IC_{50} values of antimalarials in the two categories (EDTA and TM) for both clones and the field isolates. Pearson correlation coefficient was used to determine the r values which gave the possible correlation significance of

the logarithmic values of IC_{50} between the antimalarials in clones and field isolates. Comparisons were considered significantly different at *p* values <0.05.

3.13. Ethical Considerations

Approval for the study was sought from the Jaramogi Oginga Odinga Teaching and Referral Hospital Ethics Review Committee, Appendix 6. Written informed consent, in two languages (Dholuo, and English) was given to parents or guardians of study participants (Appendix 5). Blood collection through venipuncture or finger prick can cause temporary discomfort, bruises and pain. To minimize these risks, venipuncture was only carried out by trained and qualified phlebotomist from the Ministry of Health. Besides, sterile blood collection needles were used and all sharps were stored in the appropriate biohazard sharps containers before disposal. Participation in the study was voluntary, and access to health care services was not dependent on participation. The data obtained were entered in an excel sheet in a central computer in the laboratory. For confidentiality purposes, a password was created so that data were accessed by authorized personnel handling the project.

CHAPTER 4.0: RESULTS

4.1. Introduction

This chapter contains results of the study arranged per objective and presented in tables and figures. Objective one presents the *in vitro* drug sensitivity results of the reference clones while objective two presents the sensitivity results of the field isolates in both EDTA and TM. The findings of the two objectives were correlated in the third objective in the same format.

4.2 Demographic characterictics

Among the 322 patients enrolled for the study, 206 were males and 116 were females. Among these, 65 were adults of above 19 years, 199 were schools going children between 5-18 years and 42 were children between 6 months and 4 years.

4.3 Results for objective 1.

Reference *P. falciparum* clones subjected to TM and EDTA were assayed against a panel of antimalarials namely artemether, lumefantrine, dihydroartemisinin and piperaquine. The W2 considered as chloroquine resistant, mefloquine sensitive and artemisinin sensitive and 3D7 considered as chloroquine sensitive and mefloquine sensitive were used (Desjardins *et al.,* 1979). Parasite replication across the dose range measured as Relative Flourescent Units (RFU) values of between 2000 to 4000 parasites were observed for 3D7clone and W2 had RFU values of 20,000 parasites and above. Growth inhibition by the four drugs, were calculated by an equation generating a sigmoidal concentration-response curve (variable slope), with log transformed drug concentrations on the X axis and relative fluorescent units (RFUs) on the Y axis. Curves for the RFUs across the dose ranges for both clones converged and generated sigmoidal dose-response curve variable slope with the best coefficient of determination value (r^2) of 0.9978, 0.9989 and 0.9979 among others (Figure 4.1).

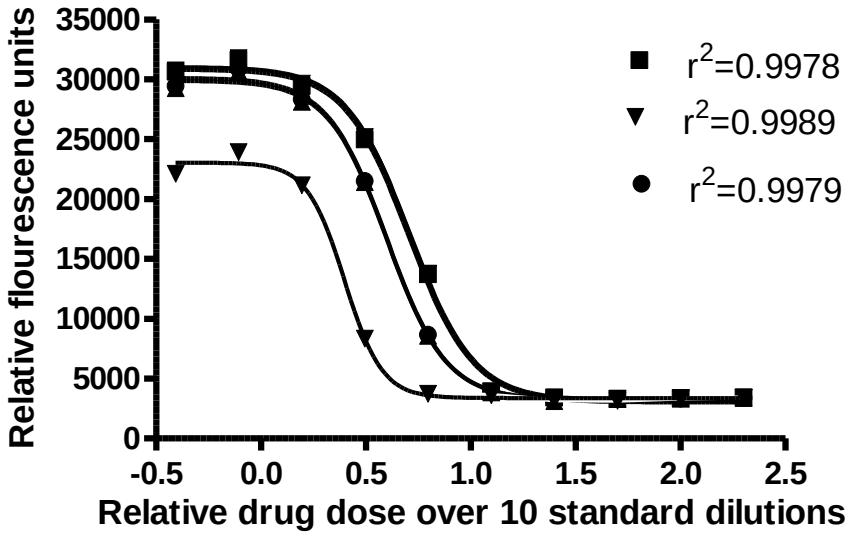

Figure 4.1: Example of sigmoidal curves with $r^2 > 0.7$

For W2 clone the IC_{50} values in ng/ml for the samples in EDTA were all higher than their respective IC_{50} values in TM as shown in Table 4.1. There was no significant difference in LUM and DHA IC_{50} values for samples stabilized in TM and EDTA, 32.61 vs 29.32 ng/ml, (p=0.369) and 18.22 vs 7.941 ng/ml, (p=0.4070) respectively. However, a high significant decrease in IC_{50} values for samples in TM was recorded when they were subjected to ART (3.118 vs1.873 ng/ml, p<0.001) and PPQ (18.93 vs 11.41 ng/ml, p=<0.001).

Table 4.1: Response of W2 reference clone in EDTA and TM against a panel of antimalarials.

		Median IC_{50} in ng/ml for W2 *P. falciparum* reference clone transported in:		
		EDTA	**TM**	
DRUGS	**n**	**Median (IQR)**	**Median (IQR)**	**p-value**
Artemether (ART)	26	3.118 (1.985-6.455)	1.873 (1.514-4.403)	<0.001
Lumefatrine (LUM)	24	32.61 (20 - 40.9)	29.32 (20.56-47.6)	0.3697
Diydroartemisinin (DHA)	20	18.22 (0.106-37.92)	14.93 (1.283-25.71.)	0.407
Piperaquine (PPQ)	26	18.93 (10.92-22.06)	11.41 (4.68-26.11)	<0.001

Comparison of the medians using Mann - Whitney test.

Key: EDTA = Ethylene Diamine Tetra Acetate, TM= Transport medium**,** n = number of samples, IQR=interquartile range, *p*= significance level of the test,

ART had the least IC_{50} values for samples in both EDTA (3.118ng/ml) and TM (1.873ng/ml). On the other hand, LUM which is the first line partner drug to ART in treatment of uncomplicated malarial had the highest IC_{50} values in EDTA (32.61ng/ml) and TM (29.32ng/ml). As seen in Figure 4.2, samples in both categories had the lowest individual IC_{50} of 20ng/ml in EDTA and 21ng/ml in TM.

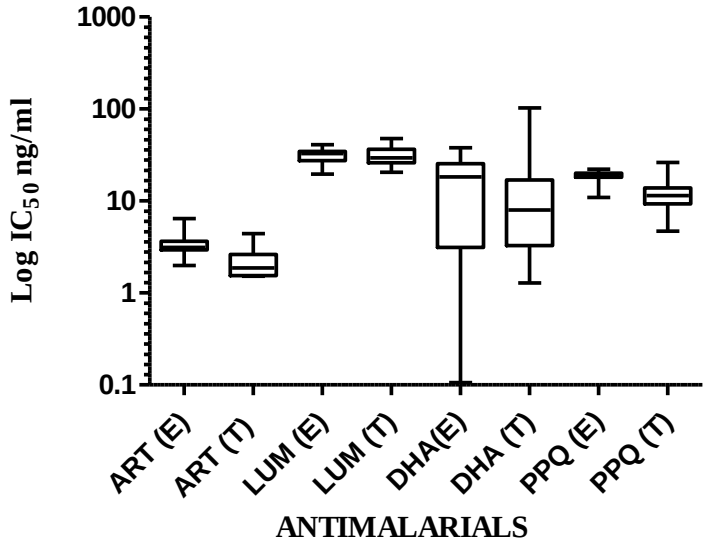

Figure 4.2: Effects of EDTA and TM on W2 reference clone

The abbreviations E and T represent samples in EDTA and Transport medium respectively, their medians IC_{50}s are in black for all the drugs

When W2 and 3D7 clones are compared, generally 3D7 clone showed reduced IC_{50} values across all the antimalarials when analyzed in either EDTA or TM. Unlike W2, samples in EDTA had lower IC_{50} values than samples in TM as shown in Table 4.2. However, there was no significant difference in IC_{50}s of ART (1.667 vs 2.353 ng/ml, p= 0.255) LUM (22.27 vs 9.905 ng/ml, p=0.3377) and DHA (2.853 vs 6.229ng/ml, p=0.0773) in the two categories. A significant decrease in IC_{50} for samples in EDTA when they were treated with PPQ (7.181 vs 12.71 ng/ml, p=0.045) was observed.

In both clones, PPQ is the only drug with a persistent significant difference in IC_{50} values (p<0.001 in W2 and p=0.045 in 3D7). As compared to W2 clone which had tight IC_{50} values, 3D7 clone showed slight variability in response to the drugs as shown in the Figure 4.3.

Table 4.2: Response of 3D7 reference clone in EDTA and TM against a panel of antimalarials

		Median IC_{50} in ng/ml for 3D7 *P. falciparum* reference clone transported in:		
		EDTA	**TM**	
DRUGS	**n**	**Median (IQR)**	**Median (IQR)**	***p*-value**
Artemether (ART)	22	1.667 (0.131- 9.09)	2.353 (0.1824-28.2)	0.2549
Lumefatrine (LUM)	24	22.27 (0.417 - 48.1)	9.905 (1.078-41.62)	0.3377
Diydroartemisinin (DHA)	20	2.853 (0.441-19.3)	10.03 (0.166-39.52)	0.0773
Piperaquine (PPQ)	23	7.181 (1.88 - 20.45)	12.71 (0.374 21.36)	0.045

Comparison of the medians using Mann - whitney test

Key: EDTA= Ethylene Diamine Tetra Acetate, TM= Transport medium**,** n= number of samples, IQR=interquartile range, *p* = significance level of the test.

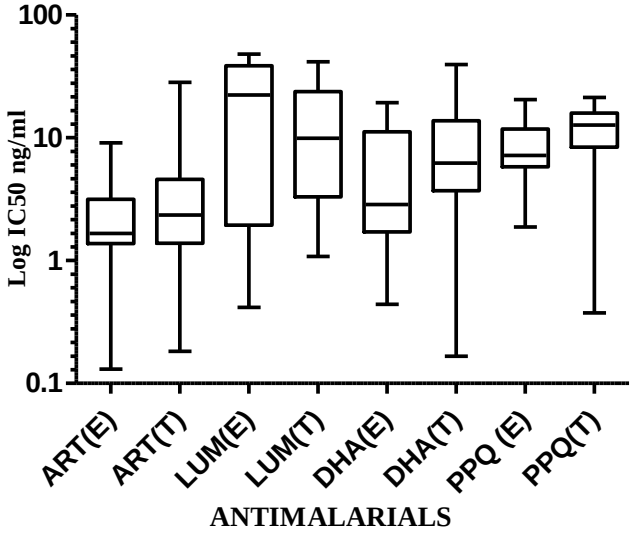

Figure 4.3: Effects of EDTA and TM on 3D7 reference clone

The abbreviations E and T represent samples in EDTA and Transport medium respectively, their medians IC_{50}s are in black for all the drugs

Correlation between effects of EDTA and TM on *in vitro* activity was determined by comparing IC_{50} values of samples in individual drugs. Results showed that there was no significant correlation (P>0.05) between the two media as indicated by the r values in Table 4.3.

Table 4.3 Correlations between EDTA and TM: Results for W2 and 3D7 reference clones.

W2 reference clones

Drug IC_{50}s in EDTA and TM	**Correlation coefficient (r)**	**Probability** **P value**
ART-ART	-0.0110	0.9611
LUM-LUM	-0.277	0.1901
DHA-DHA	-0.2577	0.2727
PPQ-PPQ	-0.1487	0.4983

3D7 reference clone

Drug IC_{50}s in EDTA and TM	**Correlation coefficient (r)**	**ProbabilityP value**
ART-ART	0.03275	0.8765
LUM-LUM	0.156	0.4771
DHA-DHA	-0.021	0.9297
PPQ-PPQ	-0.3025	0.1416

Correlation of the activity of antimalarials using Pearson's correlation coefficient of IC_{50} values.
Key: EDTA= Ethylene Diamine Tetra Acetate, TM= Transport medium**,** *P*= significance level of the test, ART= Artemether, LUM= Lumefantrine, DHA= Dihydroartemisinin and PPQ= Piperaquine

The results of the clones showed that the laboratory could analyze the samples and get read outs within expected ranges hence used the reagents and the optimized lab conditions for testing field isolates.

4.4. Results for objective 2.

A total of 322 *P. falciparum* field isolates were assayed against four panels of drugs namely ART, LUM, DHA and PPQ. Successful assay defined as a concentration-response across the 10 drug concentrations for 1 or more drugs per each *P. falciparum* field

isolate occurred in more than 50% of all the isolates in EDTA and TM. At most 70% of the samples had initial time-point zero parasitaemia >0.5% with a mean and a median of 1.03% (18-0.01%) and 0.54% respectively. At least 265 plates gave moderate RFU of between 4000-8000 parasites or even lower while 47 plates gave high relative fluorescent units (RFU) of up to 20,000 parasites for samples in both groups. When ploted against respective dose ranges, all the RFUs converged to generate dose response curves with discernible IC_{50}s at r^2 >0.7 (Figure 4.1). Those whose curves did not converge (Figure 4.4) were excluded from the study. Only 20 plates had high RFU across the plate for both low and high dose depicting possible contamination hence unused.

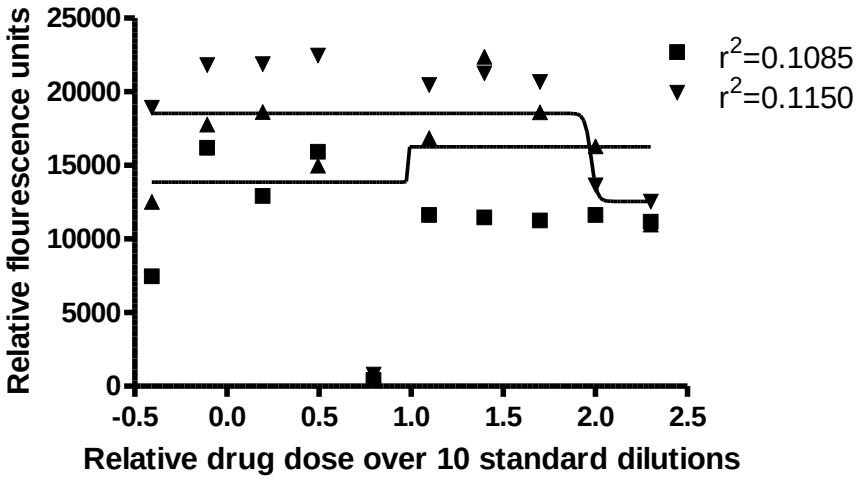

Figure 4.4: Example of sigmoidal curves with r^2 <0.7

Analysis of inhibitory concentration for EDTA or TM as summarized in table 4.4 shows that although not statistically significant, the median IC_{50} values obtained from all samples transported using EDTA anticoagulant were higher than those in TM for LUM (5.409 vs 4.007ng/ml, *p*=0.74), DHA (2.567 vs 1.691ng/ml, *p*=0.68), and PPQ (7.887 vs 6.911ng/ml, *p*= 0.82). On the other hand, the median IC_{50}s for ART in TM

and EDTA were comparable (4.887 vs 4.275ng/ml, *p*=0.99). In both categories, the least median IC_{50} values were observed for samples treated with DHA while the highest IC_{50} values were observed for those in PPQ.

Table 4.4: Response of *Plasmodium falciparum* isolates in EDTA and TM against a panel of antimalarials

		Median IC_{50} (ng/ml) for *P. falciparum* isolates transported in:		
		EDTA	**TM**	
Drugs	**n**	**Median (IQR)**	**Median (IQR)**	***p*-value**
Artemether (ART)	322	4.275(0.117-251.6)	4.887(0.226-112.1)	0.9873
Lumefatrine (LUM)	322	5.409(0.198-373.7)	4.007(0.103-190.1)	0.7448
Diydroartemisinin (DHA)	322	2.567(0.32-292.7)	1.691(0.119-401.1)	0.6839
Piperaquine (PPQ)	322	7.887(0.629-380.3)	6.911(0.516-334.8)	0.8153

Comparison of the medians using Mann - Whitney test
Key: EDTA= Ethylene Diamine Tetra Acetate, TM= Transport medium**,** *p* = significance level of the test, IQR=interquartile range, n= number of samples

Comparison of the mean IC_{50} values showed no significant different ($p>0.05$) results for the drugs in either EDTA or TM. However, samples showed a lot of variability in response to all the drugs as shown in Figure 4.5.

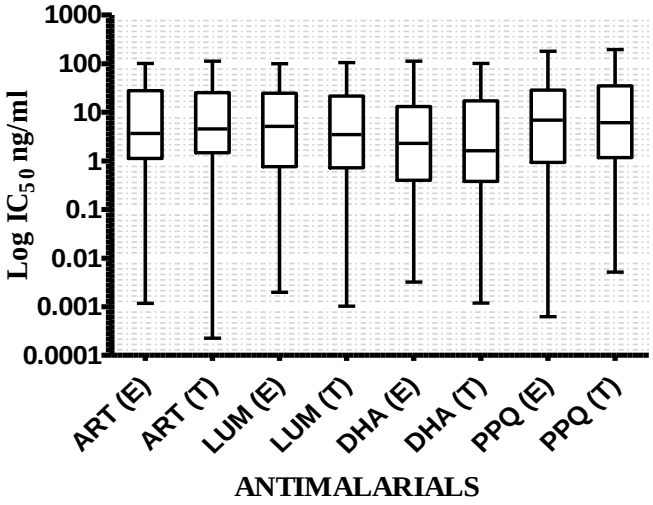

Figure 4.5: Effects of EDTA and TM on field isolates

The abbreviations E and T represent samples in EDTA and Transport medium respectively, their medians IC_{50}s are in black for all the drugs

Pearson's correlation, a measure of the linear correlation between two variables for data assuming a gaussian distribution was perfomed separately for samples which were transported in EDTA and TM as shown in Table 4.5. There was no significant correlation between samples which were transported in EDTA for ART and LUM (r=0.04967, *p*=0.37), ART and PPQ (r=0.0262, *p*=0.64), and finally LUM and PPQ (0.0584, *p*=0.29), However, significantly high correlations were observed in artemisinin derivative DHA and LUM (r = 0.1382, *p*=0.01), DHA and ART (r = 0.123, *p*=0.03) and DHA and PPQ (r = 0.1281, *p*=0.02). Among the samples in the TM, significant correlations were observed in ART and LUM (r=0.1260, *p*=0.02), ART and PPQ (r=0.1382, *p*<0.001) and finally LUM and DHA (r=0.1229, *p*=0.03) while the rest showed no significant correlation with r values lower than 0.1.

Table 4. 4 Correlation of *in vitro* responses of antimalarials drugs against field isolates of *Plasmodium falciparum* in EDTA and TM.

	***In vitro* correlation of drugs against *P. falciparum* isolates transported in:**			
	EDTA		**TM**	
Drug pairs	**Correlation coefficient (r)**	**p-value**	**Correlation coefficient (r)**	**p-value**
ART - LUM	0.04967	0.3699	0.1260	**0.0225**
ART - DHA	0.123	**0.0259**	0.09014	0.1032
ART - PPQ	0.0262	0.6364	0.1834	**0.0008**
LUM - DHA	0.1382	**0.0122**	0.1229	**0.0261**
LUM - PPQ	0.0584	0.2916	0.0758	0.1709
DHA - PPQ	0.1281	**0.0203**	0.0372	0.5024

Correlation of the drug pairs using pearsons correlation coefficient

Key: EDTA= Ethylene Diamine Tetra Acetate, TM= Transport medium, ART = Artemether, LUM = Lumefantrine, DHA = Dihydroartemisinin, PPQ =Piperaquine, *p* = significance level of the test, r = Pearson's correlation coefficient of log IC_{50} values.

4.5. Results for objective 3.

To achieve objective three, IC_{50}s obtained from each of the drugs against the clones and field isolates in both EDTA and TM were compared. Further comparison was done with the results of other published studies. Summaries of the correlates are shown in figures 4.6-4.9. In the correlation, 3D7 and W2 clones were used to guide on the range of sensitivity of the field isolates with mixed strains.

When the IC_{50} values of all the samples exposed to ART were compared in Table 4.6 and Figure 4.6, it was observed that in EDTA, the isolate had highest IC_{50} values compared to all the other samples tested (4.275ng/ml for the isolate, 1.667ng/ml for 3D7 clone and 3.118ng/ml for W2 clone). This was also observed in samples in the TM (4.887ng/ml for the isolate, 2.35ng/ml for 3D7 clone and 1.87ng/ml for W2 clone). 3D7 the chloroquine sensitive strain had least IC_{50} values in both media (1.667ng/ml in EDTA and 2.353ng/ml in TM). The W2 (CQ resistant and AR sensitive) IC_{50} values in ART were considered sensitive when compared to the results of other drugs tested. It was further observed that the IC_{50} ranges among the field isolates were

higher than the clones despite comparable medians. Their IC_{50}s were not tight with most samples above the median.

Table 4.5: Response of *P. falciparum* samples in EDTA and TM against Artemether

		Median IC_{50} (ng/ml) for samples subjected to :	
		EDTA	**TM**
Sample	**n**	**Median (IQR)**	**Median (IQR)**
Isolate	322	4.275 (0.1169-251.6)	4.887 (0.226-112.1)
3D7	22	1.667 (0.131-9.09)	2.353 (0.182-28.2)
W2	26	3.118 (1.985-6.455)	1.873 (1.514-4.403)

Key: EDTA= Ethylene Diamine Tetra Acetate, TM= Transport medium, n= number of samples, IQR= interquartile range,

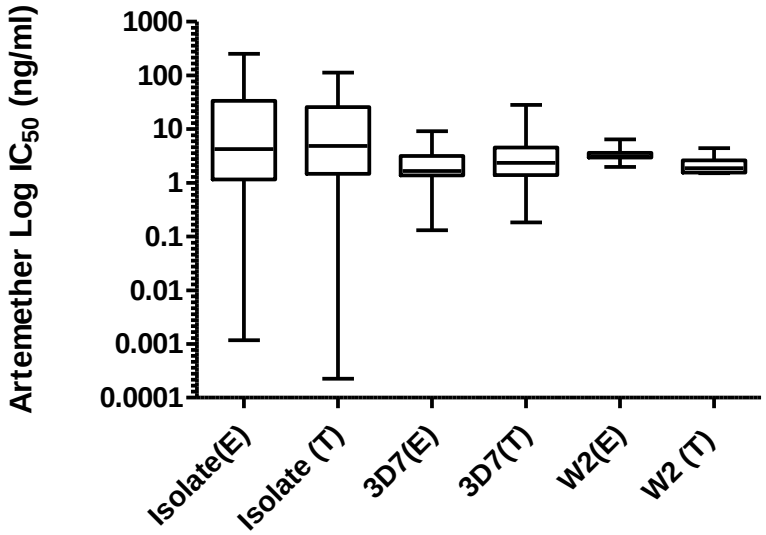

Figure 4.6: Effects of EDTA and TM on response to Artemether

The abbreviations E and T represent samples in EDTA and in Transport medium respectively. The medians IC_{50} (purple bars) with their interquartile ranges in black for all the samples.

When the samples were exposed to LUM, (table 4.7 and figure 4.7), the IC_{50} range for the isolates both in EDTA and TM were larger than for the clones, however they have remained the

most sensitive with the least IC_{50} values in EDTA (5.409ng/ml for the isolate vs 22.27ng/ml and 32.61ng/ml for the clones) and in TM (4.007ng/ml for the isolate vs 9.905ng/ml and 29.32ng/ml for the clones). More than 50% of the isolates in both categories presented with individual IC_{50} values which were lower than the median IC_{50} values of the resistant W2 clone in EDTA (32.61ng/ml) and TM (29.32ng/ml). W2 clone was the least sensitive with the highest IC_{50} values (32.61ng/ml in EDTA and 29.32ng/ml in TM) while 3D7 clone had moderate IC_{50} values (22.27ng/ml in EDTA and 9.905ng/ml in TM). Remarkably, the IC_{50} values obtained by W2 in LUM were the highest values in the whole study, recording the minimum individual IC_{50} values in both anticoagulants to be above 20ng/ml.

Table 4.6: Response of *P. falciparum* samples in EDTA and TM against Lumefantrine

		Median IC_{50} (ng/ml) for samples subjected to :	
		EDTA	**TM**
Sample	**n**	**Median (IQR)**	**Median (IQR)**
Isolate	322	5.409 (0.198-373.7)	4.007 (0.103-190.1)
3D7	24	22.27 (0.417-48.1)	29.905 (1.078-41.62)
W2	24	32.61 (20-40.9)	29.32 (20.56-47.6)

Key: Ethylene Diamine Tetra Acetate, TM= Transport medium, n= number of samples, IQR= interquartile range.

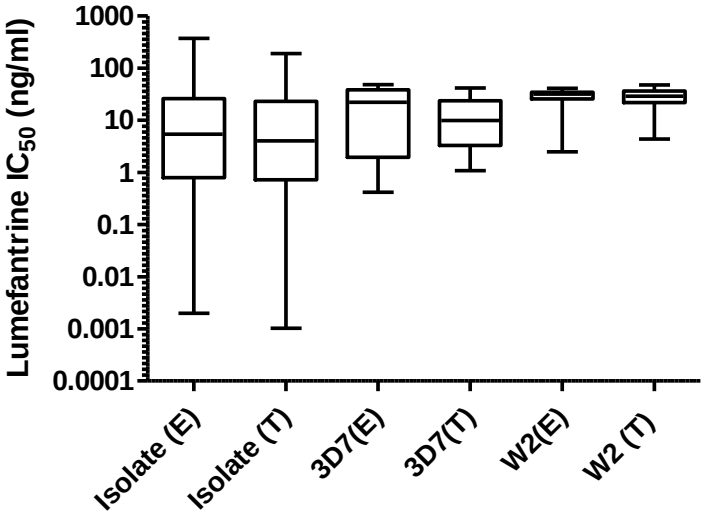

Figure 4.7 Effects of EDTA and TM on response to Lumefantrine

The abbreviations E and T represent samples in EDTA and Transport medium respectively, their medians IC_{50}s are in black for all the drugs

Further, when the samples were exposed to DHA, (table 4.8 and figure 4.8), the isolate become the most sensitive sample to DHA which is a derivative of artemisinin especially in the TM (isolate = 1.69ng/ml, 3D7 = 6.229ng/ml and W2 = 14.93ng/ml). W2 considered artemisinin sensitive had the highest values of IC_{50} to DHA compared to the isolates (1.69 and 14.93ng/ml in TM vs 2.57 and 7.941ng/ml in EDTA) an implication that the samples from this region are still sensitive to the drug. Meanwhile, the 3D7 clone considered a sensitive strain has got comparable results with the isolate especially the samples in the EDTA anticoagulant (isolate = 2.57ng/ml, 3D7 = 2.85ng/ml and W2 = 18.22ng/ml). Unlike other clones in other drugs, it was noted that there was variability in response to DHA among the clones with most IC_{50}s generally on the upper interquartile range especially for the 3D7 clone. However isolates like those in other drugs had higher IC_{50} interquartile ranges depicting variability.

Table 4.7: Response of *P. falciparum* samples in EDTA and TM against Dihydroartemisinin

		Median IC_{50} (ng/ml) for samples subjected to :	
		EDTA	**TM**
Sample	**n**	**Median (IQR)**	**Median (IQR)**
Isolate	322	2.567(0.32-292.7)	1.691 (0.119-401.1)
3D7	20	2.853 (0.441-19.3)	6.229 (0.166-39.52.09)
W2	20	18.22 (0.106-37.92)	7.941 (1.283-25.71)

Key: Ethylene Diamine Tetra Acetate, TM= Transport medium, n= number of samples, IQR= interquartile range.

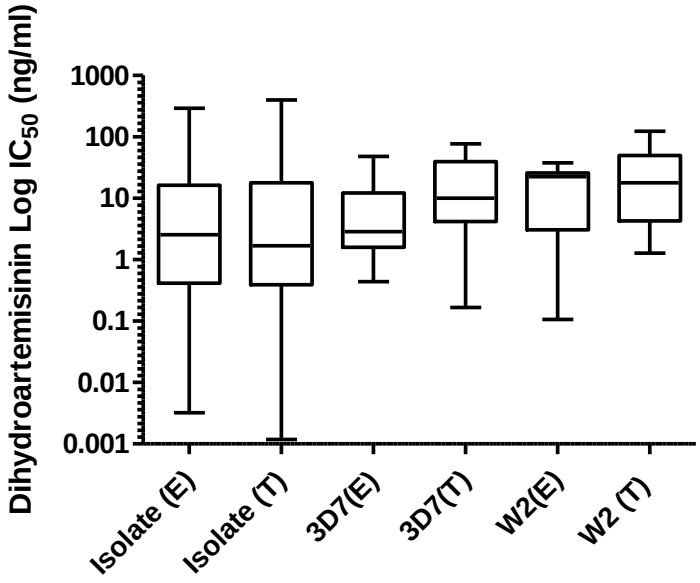

Figure 4.8: Effects of EDTA and TM on res ponse to Dihydroartemisinin

The abbreviations E and T represent samples in EDTA and Transport medium respectively, their medians IC_{50}s are in black for all the drugs

In PPQ IC_{50} distribution in figure 4.9 and table 4.9, all the clones had similar IC_{50} range, the isolates IC_{50} range were comparable despite the anticoagulants used. In EDTA W2 was the least

sensitive (18.93ng/ml) strain among them all while the isolate and 3D7clone had comparable IC_{50} values (3D7 = 7.89ng/ml vs 7.181ng/ml). In TM the isolate was still sensitive (3D7 = 12.71ng/ml vs 6.911ng/ml and W2 = 11.41ng/ml).

Table 4.8: Response of *P. falciparum* samples in EDTA and TM against Piperaquine

		Median IC_{50} (ng/ml) for samples subjected to :	
		EDTA	**TM**
Sample	**n**	**Median (IQR)**	**Median (IQR)**
Isolate	322	7.887(0.629-380.3)	6.911 (0.516-334.8)
3D7	23	7.181 (1.88-20.45)	12.71 (0.374-21.36)
W2	26	18.93 (10.92-22.06)	11.41 (4.68-26.11)

Key: Ethylene Diamine Tetra Acetate, TM= Transport medium, n= number of samples, IQR= interquartile range.

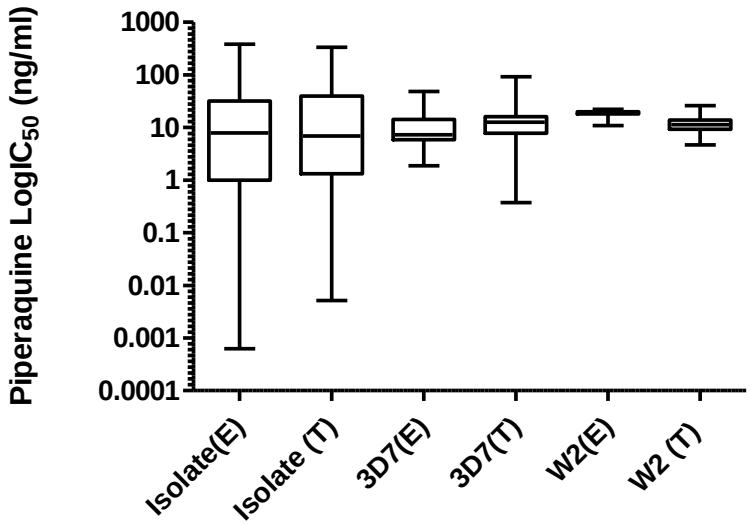

Figure 4.9: Effects of EDTA and TM on res ponse to Piperaquine

The abbreviations E and T represent samples in EDTA and Transport medium respectively, their medians IC_{50}s are in black for all the drugs

CHAPTER 5.0: DISCUSSION

5.1 Discussion for objective 1.

Among the assays on the reference clones in both anticoagulants, the IC_{50} range between the individual minimum and maximum values were narrow, this reflects reduced biological variability due to the absence subpopulations of parasites. They were able to produce distinct IC_{50} dose response curves with most points of the standard dilutions include in the sigmoidal curve. This is in accordance with a study which argued that a pure parasite population either resistant or sensitive to a drug, will produce a sigmoidal dose response curve IC_{50} values within a relatively narrow range of drug concentration, whereas a mixed population with a minor resistant population of 10% or higher will produce a flattened curve or a double-hump curve, reflecting two parasite populations with different IC_{50}. (Liu *et al.,* 2008). It has been reported that among the field isolates, most naturally occurring malaria infections are composed of mixed subpopulations with different drug susceptibilities (Postigo *et al.,* 1998; Zimmerman *et al.,* 2004). A large susceptible or resistant subpopulation may influence the apparent phenotype of the whole population (Co *et al.,* 2009), a factor relevant to the current finding.

On the other hand, W2 considered chloroquine resistant demonstrated an aspect of 'resistance' in all the drugs by having elevated IC_{50} values except for ART since its considered artemisinin sensitive. This was distinct in LUM activity with the highest median IC_{50} values than other drugs. This could have been attributed by the fact that LUM is an aminoquinoline, in the same class as CQ. Thus, observed similar trends like higher IC_{50}s for W2 than 3D7 was expected since aminoquinolines share patterns of action (Sisowath *et al.,* 2009).

The PPQ activities of 3D7 and W2 in both anticoagulants obtained in the current study were in line with the PPQ activities of 8.3ng/ml and 16 ng/ml in 3D7 chloroquine sensitive and W2 chloroquine resistant clones obtained by Vennerstrom *et al.,* (1992). It was further observed that PPQ and LUM in both clones had the least activity. This could have been attributed by the fact that the drugs are longer acting partner drugs to artemisinin derivatives with much longer half life, of 3-4 days and 3-4 weeks for LUM and PPQ respectively (German and Aweeka, 2008). Therefore, in subsequent infections

they are exposed to sub-therapeutic concentrations of these drugs, facilitating selection of parasites.

5.2. Discussion for objective 2.

In vitro drug assays in the field are best performed on fresh isolates immediately after collection (Basco, 2004). It is simpler to perform than culture adapted assays (Akala *et al.,* 2011; Bacon *et al.,* 2007) and also reduces concerns on the selection of dominant clones and loss of minor subpopulations (Liu *et al.,* 2008) hence consequent misidentification of a population's susceptibility profile (Mbaisi *et al.,* 2004). However, most malaria endemic areas are in remote locations, away from established laboratories. On the contrary, malaria drug testing labs are few which are specialized and located mostly in urban areas. These two factors present potential ability in handling of sample prior to performance of assay. For some laboratories, blood samples containing *P. falciparum* parasites for testing may be stored at collection sites for days or transported long distances to the laboratory before cultivation (Akala *et al.,* 2011). This factor could cause failure of growth attributed to a prolonged time between the times when samples were collected and when they were tested (Bacon *et al.,* 2007) hence reducing the parasites viability to even zero (Basco, 2004).

Availability of a medium would provide a proper transition from the natural host to an artificial medium and would keep the parasites viable to the laboratory for cultivation. In that regard, this study employed the use of a formulated transport media (TM) with an aim of trying to improve on the viability of parasites during transportation to the laboratory for further cultivation. This is the first study to report a direct comparison between two anticoagulants, evaluating the possible influence of TM on maintaining parasite viability during storage at 4°C against the conventional EDTA anticoagulant. The EDTA has the advantages of a lack of inhibitory action on DNA

allowing the best preservation of cellular components and morphology of blood cells (Reardon *et al.,* 1993), thus best for biological effect, hematologic and biochemical investigations (Basco, 2004).

Among the field isolates collected, it was noted that initial time point zero parasitaemia for 70% of the samples (n=322) was >0.5% with a mean of 1.03%. This concur with a Kenyan study which reported that 88% of the field isolates collected (n=292) in 2008-2009 had parasitaemia of >0.2% (Chaorattanakawee *et al.,* 2013). Natural infections usually have different levels of parasitemia at the time individuals present at hospitals with symptoms for medical care. This difference is occassioned by varying host immunity and parasite virulence that modulate diesease progression leading to hospital seeking behavior (Langhorne *et al.,* 2008).

Variation in starting parasitemia in *in vitro* drug susceptibility tests has been shown to alter IC_{50}s (Liu *et al.,* 2008). Parasitaemia estimation in any *in-vitro* assay is highly dependent on microscopy therefore, variation occurs depending on the expertise of the microscopist in the laboratory. If the starting parasitaemia in an assay is mistakably high then much infected parasites would be found in each well of the plate leading to an increase in the absorption of drugs, (Ritchie *et al.,* 1996) consequently increasing the IC_{50} levels obtained (Geary *et al.,* 1990). This partly explains the high levels of RFU when curves were plotted against the drug concentration in the present study. The reverse side, a very low starting parasitaemia may lead to a lack of growth and less IC_{50} values (Bacon *et al.,* 2007). This factor is important as studies transition to immediate *ex vivo* analysis of field isolates.

Among the assays, the IQR of the IC_{50} was high in our study for all the drugs in both categories, which may reflect greater biological variability among the field isolates with the presence of sub

populations with varying drug response (Jefari *et al.,* 2004). This could be one of the reasons why 13% of the samples generated curves with undetermined IC_{50}s. The data points scattered across the plot could not converge to create sigmoidal dose-response curve. Similar results have been shown in laboratory condition using clones with known responses. Co and coworkers (Co *et al.,* 2009) analyzed mixture varying proportions of reference clones that have distict difference in IC_{50}s to mimic subpopulations inherent of field isolates. This study observed that presence of a subpopulation proportions less than 10% of the sample alters the the shape of the curve to bimodal (Co *et al.,* 2009).

Increasing the number of infected erythrocytes lead to requirement of higher drug concentration to attain optimal effect (Basco, 2004) proportionate to the numbers of parasites per volume (Duraisinngh *et al.,* 1999; Gluzman *et al.,* 1987). A study in Cambodia also found that parasitaemia level is inversely proportional to the IC_{50} values (Chaorattanakawee *et al.,* 2013). Parasitemia determination in this study was by microscopy. Though this is the gold standard method, it has been shown to be highly subjective and dependent of the technician. Different microscopists were involved in parasitemia analysis. It is probable that this subjectivity contributed to the high IC_{50} values obtained with 4% of the plates which consequently raised the mean and median IC_{50} values especially for PPQ in the current study for samples in both EDTA and TM since both used the same amount of parasitaemia.

The DHA was the most active drug with the least median IC_{50} values (2.567ng/ml in EDTA and 1.691ng/ml in TM) and ranges in both EDTA and TM samples although were not significant. This could be attributed by the fact that reduced artemisinin susceptibility has not been reported in the region and generally in Kenya. This is in line with a study conducted at the Kenyan coast in which DHA emerged the most active drug (Mwai *et al.,* 2009; Achieng *et al.,* 2015).

Highest IC_{50} values of 7.887ng/ml in EDTA and 6.911ng/ml in TM samples were obtained by PPQ. These values were lower than IC_{50} values from previous studies done in malaria endemic areas (Basco *et al.,* 2003; Basco *et al.,* 2007 and Mwai *et al.,* 2009). PPQ has not yet been deployed in Kenya for malaria treatment. These results suggest that it is safe to deploy PPQ containing regimens for treatment of malaria in Kenya. However, molecular studies, profiling markers of PPQ resistance need to be instituted on these samples and compared with literature in order to clarify the observed response.

In contrast with other studies which have found an IC_{50} LUM activity of 15ng/ml for more than 95% of isolates (Mayxay *et al.,* 2007; Parola *et al.,* 2007; Paradines *et al.,* 2006), the current study generated only 24% and 20% from samples in EDTA and TM respectively.This is a clear indication that the activity of the drug which is used as a partner to ART for treatment of uncomplicated malaria cases remains high in the region.

ART IC_{50} data which were 4.27ng/ml in EDTA and 4.88nm in TM was comparable with those found earlier for clinical isolates collected in Cameroon and other African countries. They reported IC_{50} values range between 3.46ng/ml and 5.66ng/ml (Basco and LeBras, 1993; Basco and Ringwald, 2003 and Issaka *et al.,* 2013). These concur with a Kenyan study where, there has been unchanged susceptibility to ART as reported by Akala *et al.,* (2011) on Kenyan western samples collected one year after commencement of ACT policy. Later, Eyase *et al.,* (2013) reported, and Achieng *et al.,* (2015) for samples collected five and eight years respectively after deployment of ACTs. This unchanged susceptibility to ART suggests that treatments comprisisng this regimens are still effective in Kenya.

The inoculum size used in *in vitro* testing influences the measured *in vitro* susceptibility to antimalarials; therefore resistance can be overestimated when inoculum effects are not

considered. Occurrences of cross resistance between antimalarials can also be falsely determined in some cases (Duraisinngh *et al.,* 1999). Positive correlation was observed between DHA and all the tested drugs (ART, LUM and PPQ) for samples in EDTA, unlike the same samples in TM where DHA had statistically significant correlation of r=0.1229 (p=0.02) with LUM only while others were not significant. The variation observed in cross resistance of DHA in the samples in EDTA and TM would have been attributed by the inoculum effect. However, further research need to be conducted to rule out the possible cross resistance between these drugs in the region. A study at the coast in Kenya found a positive correlation between DHA and LUM, as well as PPQ and LUM (Mwai *et al.,* 1999), thus in line with the results of the current study.

5.3 Discussion for objective 3.

Artemisinin-based combination therapies (ACTs) have shown excellent efficacy and are now recommended to treat falciparum malaria in nearly all countries (WHO 2010). ACTs include potent, short acting artemisinins that rapidly reduce parasite biomass and alleviate malaria symptoms and longer acting partner drugs that improve antimalarial efficacy and reduce the risk of selection for artemisinin resistance (Nosten and White, 2007). This could explain the high sensitivity to artemisinin derivatives as compared to their partner drugs in the current study for both the field isolates and the reference clones and in another study (Zongo *et al.,* 2007).

Artemether-lumefantrine (AL) is the most widely recommended ACT in Africa (Sinclair *et al.,* 2009). ACTs were adopted as first-line treatment for uncomplicated malaria in many African countries in 2006, including Kenya (Kokwaro *et al.,* 2007) where it has shown outstanding efficacy (Dorser *et al.,* 2010). Studies have also confirmed its high success rates of 90 to 95% in many areas where malaria is endemic including Kenya (Falade *et al.,* 2008, Kobbe *et al.,* 2008, and Yeka *et al.,* 2008). These concur with the results of LUM in the current study

where the median IC_{50} values of 5.4ng/ml in EDTA and 4ng/ml in TM were the least ever reported.

The *in vitro* activity of LUM against field isolates from several areas where malaria is endemic has been investigated using the WHO microtest, and in all these studies LUM activity was high with IC_{50} of 15ng/ml for more than 95% of isolates (Anderson *et al.,* 2005, Pradines *et al.,* 2006, Palora *et al.,* 2007, Basco and Ringwald, 2007, Maxxay *et al.,* 2007, Kaddouri *et al.,* 2008). A similar study which was perfomed and observed even higher IC_{50} range and IC_{50} values of 106ng/ml for most isolates (Mwai *et al.,* 2009) were in agreement with the report of a Senegalese study (Pradines *et al.,* 1999). Therefore, LUM is still effective in malarial treatment in the region.

This study also generated the sensitivity data for ART and found that the field isolates were not as sensitive as the artemisinin sensitive W2 reference clone and 3D7 clone, a worrying issue at hand, suggesting that the drug should be monitored and used rationally. In addition, despite the comparable medians the IC_{50}s among the isolates were not tight with most samples above the median. This depicts a high variablility with response to the drug. Nevertheless, the interpretation of these results should also consider the weaknesses of the standard *ex vivo* tests and other methodological variability. But then again, this study results were also higher than the results of a previous Cameroonian study whose IC_{50} value was 3.71ng/ml (Basco and Lebras 1993) but in accordance with other studies carried out in sub Saharan Africa (Fall *et al.,* 2011, Pascual *et al.*, 2012).

Introduction of PPQ for use as a monotherapy in Africa is not recommended due to the rapid emergence of clinical resistance observed in China (Lan *et al.,* 1989, Chen, 1991, and Guo *et al.,* 1993). In that case PPQ and DHA have been chosen as partner drugs as a second line treatment

of uncomplicated malaria in Kenya (WHO, 2010). Our results suggest that PPQ and DHA are equally active against the field isolates as compared to the sensitive and the resistant reference clones. In a study on field isolates from Madagascar (Deloron *et al.,* 1985) the IC_{50} of PPQ were widely dispersed ranging from 12.5 to 250ng/ml with values of 100ng/ml for majority (83%) of isolates. By contrast, this study showed moderate level of activity of PPQ within similar range but with IC_{50} of 100ng/ml for only 14% in EDTA and 10 % in TM. However, a higher level of activity was demonstrated by a Cameroonian study, with all the isolates within a narrower range of 7.76 to 78.3ng/ml. (Leonardo and Pascal, 2003).

PPQ and LUM as partner drugs demonstrated a good activity with field isolates in this study although they showed the least activity with high median IC_{50} values compared to the artemisinin derivatives (ART and DHA) drugs. This could have been attributed by the fact that they have a longer half life (3-5 days for LUM) and much longer (3-4 weeks for PPQ) than other ACT partner drugs (German and Aweeka, 2008).This facilitates selection of parasites with reduced sensitivity to the partner drugs such that in case of subsequent infections, parasites will be exposed to subtherapeutic concentrations of the drugs, a factor which can easily lead to reduce susceptibility and even resistance to the drugs (Melissa *et al.,* 2014). This selection is more in PPQ which has a longer half-life than LUM. PPQ however, benefits from the activity of DHA as a partner drug which has shown excellent efficacy in Africa (Kamya *et al.,* 2007, Yeka *et al.,* 2008, Arinaitwe *et al.,* 2009) and even in the current study. DHA sensitivity results were much lower than the results of W2 clone (considered AR sensitive) but comparable with other *ex vivo* field isolates studies (Nsobya *et al.,* 2010, Lim *et al.,* 2013). Subsequently, PPQ resistance does not appear to be a major problem and therefore, DHA and PPQ combination has been adopted as a first line therapy in South East Asia (WHO, 2010).

On the field isolates, DHA showed the best activity of 2.567 ng/ml in EDTA and 1.691 ng/ml in TM. These values were below the values of both reference clones in the current study although higher than the results obtained in a study (Basco, 2003). This study generated DHA IC_{50} median of 1.29ng/ml for the field isolates against a 1.12 ng/ml of CQ sensitive and 1.39ng/ml of CQ resistant clones and in another study in Burkina Faso (Halidou *et al.,* 2014). The activity of DHA demonstrated by the current study isolates was below the cut off of 10.5ng/ml used in a study in Congo (pradines *et al.,* 1998) and in the Kenyan Coast (Mwai *et al.,* 2009) but comparable with other studies (Yavo *et al.,* 2009; Hao *et al.,* 2013).

A marginal statistically significant correlation observed between PPQ and DHA in EDTA samples (r= 0.1281; *p*= 0.0203) and no correlation in TM samples (r= 0.0372; *p*= 0.5024) was encouraging because at this low coefficient correlation level, *in vitro* cross-resistance is unlikely to occur. These data were in accordance to the previous reports (Nsobya *et al.,* 2010) and another study which found absence of cross resistance due to the absence of association between PPQ and genes involved in quinolone resistance (Briolant *et al.,* 2010). Thus, a reassurance for sustained use of the drug which is inexpensive, safe and a highly effective treatment for uncomplicated *P. falciparum* and *P. vivax* malaria (Price *et al.,* 2007, Myint *et al.,* 2007, Awab *et al.,* 2010). Besides, it offers a better post treatment prophylatic effect following therapy compared with Artemether Lumefantrine (Kamya *et al.,* 2007; Zongo *et al.,* 2007; Yeka *et al.,* 2008).

CHAPTER 6.0. SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1. SUMMARY

These results highlighted no significant difference in the TM and EDTA when subjected to the clones. Field isolates have also shown no significant different results, however, it was apparent that the values obtained by the samples in EDTA were higher than those for the TM a feature which could depict methodological variability. Correlation coefficient was obtained by DHA and other drugs tested in both EDTA and TM a feature which would depict cross resistance between these drugs. This requires further research to elucidate on the finding. On comparison of the field isolates and clones, it was found out that, the field isolates' IC_{50} values were comparable to the sensitive 3D7 clone than to W2 clone. These results were also comparable to the other published findings.

6.2 CONCLUSION

In conclusion,

1. The clones in TM have generally shown no significant difference in sensitivity results with the clones exposed to the TM.
2. The comparable *in vitro* effect in the field isolates samples which were in the EDTA and TM suggests that TM equally preserves *P.falciparum* infected cells
3. The field isolates results shows that population of Maseno Division infected with *P. falciparum* are still responsive to the tested drugs. Importantly, the results were comparable to the CQ sensitive 3D7 clone than CQ resistant W2 clone.

6.3 RECOMMENDATIONS

This study recommends that:

1. Sensitivity studies should be done across other standard clones to establish their patterns using the proposed TM.
2. Once the response has been confirmed in large sample sets and other laboratory settings the TM can be considered for commercialization.
3. Tests should be done on a larger number of field isolates in order to confirm observed findings.

6.4 SUGGESTION FOR FUTURE STUDIES

It is important to do a study to compare the storage time and efficacy of the TM in the viability of *P. falciparum* positive blood cells.

This study also suggests that artemisinin derivatives are still very efficacious and dihydroartemisinin piperaquine seems a valuable alternative ACT. Variations in the positive correlations experienced by the drug pairs in TM and EDTA anticoagulants is possibly due to cross resistance among them. This requires further research to elucidate on the finding.

REFERENCES

Achieng, A.O., Muiruri, P., Ingasia, L.A., Opot, B.H., Juma, D.W., Yeda, R., Ngalah, B.S. Ogutu, B. R., Ben. Andagalu, Akala, H.M., Edwin, Kamau, E. (2015). Temporal trends in prevalence of *Plasmodium falciparum* molecular markers selected for by artemetherelumefantrine treatment in pre-ACT and post-ACT parasites in western Kenya. *International journal for parasitology: drugs and drug resistance*: 5: 92-99

Abdo-Rabbo, A. (2003). Household survey of treatment of malaria in Hajjah, Yemen. *Eastern Mediterranean Health Journal.* 9**:** 600-606.

Akala, H.M., Eyase, F.L., Cheruiyot, A.R., Omondi, A.A., Ogutu, B.R., Waters, N.C., Johnson, J.D., Polhemus, M.E., Schnabel, D.C., Walsh, D.S. (2011): Anti-malarial drug sensitivity profile of western Kenya *Plasmodium falciparum* field isolates determined by a SYBR Green I *in vitro* assay, and molecular analysis. *American Journal of Tropical medicine and hygiene* 85*:* 34 – 41.

Amin, A. A., Walley, T., Kokwaro, G. O., Winstanley, P. A., and Snow, R. W. (2007). Commentary: Reconciling national treatment policies and drug regulation in Kenya. . *Health Policy Planning,* 22: 111-112.

Anabwani, G. M., Esamai, F. O. and Menya, D. A. (1996). A randomised controlled trial to assess the relative efficacy of chloroquine, amodiaquine, halofantrine and fansidar in the treatment of uncomplicated malaria in children. *East African Medical Journal,* 73: 155-158.

Anders, R. F. and Smythe, J. A. (1989). Polymorphic antigens in *Plasmodium falciparum*. *Blood*. 74: 1865-1875.

Anderson, T. J., Haubold, B., Williams, J. T, Estrada-Franco Section Sign JG, Richardson L, Mollinedo R, Bockarie M, Mokili J, Mharakurwa S, French N, Whitworth J, Velez ID, Brockman AH, Nosten F, Ferreira MU, Day KP, (2000). Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium falciparum. Molecular Biology and Evolution* 17*:* 1467–1482.

Anderson, T. J., S. Nair, H. Qin, S. Singlam, A. Brockman, L. Paiphun, and F. Nosten. (2005). Are transporter genes other than the chloroquine resistance locus (*pfcrt*) and multidrug resistance gene (*pfmdr*) associated with antimalarial drug resistance? *Antimicrobial Agents Chemotherapy*. 49:2180–2188.

Arinaitwe, E., Sandison, T. G., Wanzira, H. (2009). Artemether-lumefantrine versus dihydroartemisinin-piperaquine for *falciparum* malaria: a longitudinal, randomized trial in young Ugandan children. *Clinical Infectious Disease.* 49:1629–37

Awab, G .R., Pukrittayakamee, S., Imwong, M., Dondorp, A. M., Woodrow ,C. J., Lee, S. J, Day, N. P. J., Singhasivanon, P. White, N. J., Kaker, F. (2010). Dihydroartemisinin piperaquine versus chloroquine to treat vivax malaria in Afghanistan: an open randomized, non-inferiority, trial. *Malaria Journal*. 9:105

Awad, A., Eltayeb, I., Matowe, L. and Thalib, L. (2005). Selfmedication with antibiotics and antimalarials in the community of Khartoum State, Sudan. *Journal of Pharmacy and Pharmaceutical Sciences.* 8: 326-331.

Babiker, H. A., Creasy, A. M., Bayoumi, R. A. L., Walliker, D. and Andarnot, D. E. (l991b). Genetic diversity of *Plasmodium falciparum* in a village in Eastern Sudan. Drug resistance, molecular karyotypes and the mdr-1 genotype of recent isolates. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. 85: 578-583

Bacon, D. J., Latour, C., Lucas, C., Colina, O., Ringwald, P., & Picot, S. (2007). Comparison of a SYBR green I-based assay with a histidine-rich protein II enzyme-linked immunosorbent assay for *in vitro* antimalarial drug efficacy testing and application to clinical isolates. *Antimicrobial Agents Chemotherapy, 51*, 1172-1178.

Baicu, S.C., and Taylor, M.J. (2002). "Acid-base buffering in organ preservation solutions as a function of temperature: new parameters for comparing buffer capacity and efficiency". *Cryobiology:* 45 (1): 33–48

Baniecki, M. L., Wirth, D. F., and Clardy, j. (2007). High-throughput *Plasmodium falciparum* growth assay for malaria drug discovery. . *Antimicrobial Agents Chemotherapy, 51*, 716-723.

Basco L.K., La Bras J, (1993a). *In vitro* activities of monodesethyLamodiaquine and amopyroquine against African isolates and clones of *Plasmodium falciparum*. *American Journal of Tropical Medicine and Hygiene* 48:120-125.

Basco L.K, La Bras, J. (1993b). *In vitro* activity of artemisinin derivatives against African isolates and clones of *Plasmodium falciparum*. *American Journal of Tropical Medicine and Hygiene.* 49: (3), 301-307.

Basco, L. K. (2002). Molecular epidemiology of malaria in Cameroon. XIII. Analysis of pfcrt mutations and *in vitro* chloroquine resistance. *American Journal of Tropical Medicine and Hygiene,* 67: 388-391.

Basco, L. K. (2003). Molecular epidemiology of malaria in cameroon.xv. Experimental studies on serum substitutes and supplements and alternative culture media for *in vitro* drug sensitivity assays using fresh isolates of *plasmodium falciparum*. *American Journal of Tropical Medicine and Hygiene,* 69(2): 168–173.

Basco, L. K. (2004). Molecular epidemiology of malaria in cameroon. xx. Experimental studies on various factors of *in vitro* drug sensitivity assays using fresh isolates of *plasmodium falciparum*. *American Journal of Tropical Medicine and Hygiene,* 70(5), 474–480.

Basco, L. K., and Le Bras, J. (1990). Reversal of chloroquine resistance with desipramine in isolates of *Plasmodium falciparum* from Central and West Africa. *Transactions of the Royal Society of Tropical Medicine and Hygiene.* 84:479–481.

Basco, L. K., and Le Bras, J. (1993). *In vitro* susceptibility of Cambodian isolates of *Plasmodium falciparum* to halofantrine, pyronaridine, and artemisinin derivatives. *Annals of Tropical medicine and parasitology*. 88:137–144.

Basco, L. K., and P. Ringwald. (2003). *In vitro* activities of piperaquine and other 4-aminoquinolines against clinical isolates of *Plasmodium falciparum* in Cameroon. *Antimicrobial Agents Chemotherapy* 47:1391–1394.

Basco, L. K. and Ringwald, P. (2007). Molecular epidemiology of malaria in Cameroon. XXIV. Trends of *in vitro* antimalarial drug responses in Yaounde, Cameroon. *Americal Journal of Tropical Medicine and Hygiene.* 76:20–26.

Bendixen, M., Msangeni, H. A., Pedersen, B.V., Shayo, D., Bodker, R. (2001). Diversity of *Plasmodium falciparum* populations and complexity of infections in relation to transmission intensity and host age: a study from the Usambara Mountains, Tanzania. *Transactions of the Royal Society of Tropical Medicine and Hygiene 95:* 143–148.

Bennett, T. N., Paguio, M., Gligorijevic, B., Seudieu, C., Kosar, A. D., Davidson, E., & Roepe, P. D. (2004). Novel, rapid and inexpensive cellbased quantification of antimalarial drug efficacy. *Antimicrobial Agents Chemotherapy. 48*: 1807-1810.

Bickii, J., Basco, L. K., & Ringwald, P. (1998). Assessment of three *in vitro* tests and an in vivo test for chloroquine resistance in *Plasmodium falciparum* clinical isolates. *Journal Clinical Microbiology. 36*, 243-247.

Bjorkman, A., and Bhattarai, A. (2005). Public health impact of drug resistant *Plasmodium falciparum* malaria. *Acta Tropical, 94*, 163-169.

Björkman, A., Willcox, M. (1986) *In vivo* and *in vitro* susceptibility of *Plasmodium falciparum* to sulphadoxine/pyrimethamine in Liberia, West Africa. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 80: 572–574.

Bloland, P. (2001). Drug Resistance in Malaria. *World Health Organization Geneva. WHO/CDS/DRS, 4*, 2.

Borrmann, S., Sasi, P., Mwai, L., Bashraheil, M., Abdallah, A., Muriithi, S., Frühauf, H., q Schaub, B., P. feil, J., Peshu, J., Hanpithakpong, W., Rippert, A, Juma, E., Tsofa, B., Mosobo, M., Lowe, B., Osier, F., Fegan, G., Lindegårdh, N., Nzila, A., Peshu, N., Mackinnon, M., Marsh, K. (2011). Declining responsiveness of *Plasmodium falciparum* infections to artemisinin-based combination treatments on the Kenyan coast. *PLoS ONE*, 6:e26005.

Bottenstein, J., Hayashi, I., Hutchings, S., Masui, H., Mather, J. and McClure, D. (1979). The growth of cells in serum-free hormone-supplemented media. *Methods in Enzymology.* 58:94- 109.

Brasseur, P., Kouamouo, J., Moyou-Somo, R., Druilhe P. (1992). Multi-drug resistant falciparum malaria in Cameroon in 1987-1988. I. Stable figures of prevalence of chloroquine and quinine-resistant isolates in the original foci. *American Journal of Tropical Medicine and hygiene.* 46: 47.

Breman, J. G., Alilio, M. S., and Mills, A. (2004). Conquering the intolerable burden of malaria: what's new, what's needed: a summary. *American Journal of Tropical medicine and hygiene. 71,* 1-15.

Briolant, S., Henry, M., Oeuvray, C., Amalvict, R., Baret, E., Didillon, E., Rogier, C., Pradines, B. (2010). Absence of association between piperaquine *in vitro* responses and polymorphisms in the pfcrt, pfmdr1, pfmrp,

Bruce-Chwatt, L. J., Black, R. H., Canfield, C. J., Clyde, D. F., Peters, W. (1984). Chemotherapy of malaria. (2nd ed. World.).

Brumpt, E. (1999). The human Parasites of the genus *Plasmodium*. In "Malariology: A Comprehensive Survey of All Aspects of This Group of Diseases from a Global Standpoint" (M. F. Boyd, Ed.), pp. 65-121. Saunders, Philadelphia/London.

Buabeng, K. O., Duwiejua, M., Dodoo, A. N., Matowe, L. K. and Enlund, H. (2007). Self-reported use of antimalarial drugs and health facility management of malaria in Ghana. *Malaria Journal.* 6**,** 85-90.

Caligaris, S., Fadat, G., Matteelli, A., Castelli, F., Kouka Bemba, D., Carosi, 0., (1992). Etudede la chimiosensibilite de *Plasmodium falciparum* aux antimalariques dans Ia ville deYaounde (Cameroun). *Bulletin of the Society of Pathologie Exotique.* 85: 279-280

Cavill, I., Kraaijenhagen, R., Pradella, R., D'Onofrio, G., Herkner, K., Rowan, R.M. (1996). *In vitro* stability of the reticulocyte count. *Clinical and Laboratory Haematology Journal*. 18: 9–11.

Chaorantanakawee, S., Stuart, D. T. Chanthap, L., Kritsanai, Y., Wiriya, R.,S., Sundrakes, P. S., Jacob D. Johnson, Douglas S. Walsh, David L. Saunders and Charlotte A Lanteri. (2013). Direct comparison of the histidine-rich protein-2 enzyme-linked immunosorbent assay (HRP- 2ELISA) and malaria SYBR green I fluorescence (MSF) drug sensitivity

tests in Plasmodium falciparum reference clones and fresh ex vivo field isolates from Cambodia. *Malaria Journal* 12:239.

Chen, L. (1991). Recent studies on antimalarial efficacy of piperaquine hydroxypiperaquine. *Chinese Medical Journal*. 104:161–163

Cheruiyot, A.C., Auschwitz, J.M., Lee, P.J., Yeda, R.A., Okello, C.O., Leed, S.E., Talwar, M., Murthy. T., Gaona, H.W., Hickman, M.R., Akala, H.M., Kamau, E., Johnson, J.D. (2016). Assessment of the Worldwide Antimalarial Resistance Network standardized procedure for *in vitro* malaria drug sensitivity testing using SYBR green assay for field samples with various initial parasitemia levels. *Antimicrobial Agents Chemotherapy* 60:2417–2424.

Chevli, R., Fitch, C.D, (1982). The antimalarial drug mefloquine binds to membrane phospholipids. *Antimicrob Agents Chemother*. 21: 581-586.

Childs, G. E., Wimonwattrawatee, T., & Pooyindee, N. (1988). Evaluation of an *in vitro* assay system for drug susceptibility of field isolates of *Plasmodium falciparum* from southern Thailand. *American Journal of Tropical medicine and hygiene, 38*: 19-23.

Chulay, J. D., Haynes, J. D., Diggs, C. L. (1983). *Plasmodium falciparum*: assessment of *in vitro* growth by [^{3}H] hypoxanthine incorporation. *Experimental parasitology*, 55: 138–146.

Cinatl, J. (1969). Inorganic-organic multimolecular complexes of salt solutions, culture media and biological fluids and their possible significance for the origin of life. *Journal of Theoretical Biology*. 23:1-10

Co EM, Dennull, R.A., Reinbold, D.D., Waters, N.C., Johnson, J.D., (2009). Assessment of malaria *in vitro* drug combination screen- ing and mixed-strain infections using the malaria Sybr green I-based fluorescence assay. *Antimicrob Agents Chemotherapy*. 53*:* 2557–2563.

Creasy, A., Fenton, B., Walker, A., Thaithong, S., 0liveira, S., Mutambu, S. and Walliker, D. (1990). Genetic diversity of *Plasmodium falciparum* shows geographical variation. *American Journal of Tropical Medicine and Hygiene*. 42, 403-413

Dabis F., Murphy, K., Jones, T.S. and Karsa, T. (1999).Monitoring selective components of primary health care: methodology and community assessment of vaccination, diarrhoea, and malaria practices in Conakry, Guinea. *Bulletin of the World Health Organization*, 67: 675-684.

Darfler F. (2000). A protein-free medium for the growth of hybridomas and other cells of the immune system. *In Vitro Cellular and Developmental Biology.* 26:769-78.

Davis, J. M., edition. (1994) Basic Cell Culture: A Practical Approach New York: Oxford University Press, 1994: 59-60.

De Monbrison, F., Raynaud, D., Latour-Fondanaiche, C., Staal, A., Favre, S., Kaiser, K., S, P. (2003). Real-time PCR for chloroquine sensitivity assay and for *pfmdr1Plasmodium falciparum*. *Journal of Microbiological Methods, 54*(3), 391-401.

Deloron, P., J. Le Bras, J. A. Ramanamirija, and P. Coulanges. (1985). *Plas modium falciparum* in Madagascar: *in vivo* and *in vitro* sensitivity to seven drugs. *Annals of Tropical Medicine and Parasitology.* 79:357–365

Deming, M.S. (2004). Home treatment of febrile children with antimalarial drugs in Togo. *Bulletin of the World Health Organization*. 67: 695-700.

Deming, M.S., Gayibor, A., Murphy, K., Jones, T.S. and Karsa, T. (1998). Home treatment of febrile children with antimalarial drugs in Togo. *Bulletin of the World Health Organization.* 67, 695-700.

Deressa, W., Ali, A. and Enqusellassie, F. (2003). Self-treatment of malaria in rural communities, Butajira, southern Ethiopia. *Bulletin of the World Health Organization.* 81: 261-268.

Desjardins, R. E., Canfield, C. J., Haynes, J. D., and Chulay, J. D. (1979). Quantitative assessment of antimalarial activity *in vitro* by a semiautomated microdilution technique. *Antimicrobial Agents Chemotherapy, 16,* 710-718.

Di Perri, G., Olliaro, P., Nardi, S, Deganello, R, Allegranzi, B., Bonora, N S., Vento, S., Concia, E (1998). Response of uncomplicated falciparum malaria to oral chloroquine and quinine in Burundi Highlands. *Acta Trop.* 70: 25-33.

Dorsey, G., Kamya, M. R., Singh, A., Rosenthal, P. J. (2011). Four Artemisinin-Based Combinations Study Group. A head-to-head comparison of four artemisinin-based combinations for treating un-complicated malaria in African children: a randomized trial. *PLoS Medicine* 8:1001-1119.

Druilhe, P., Moreno, A., Blanc, C., Brasseur, P. H., and Jacquier, P. (2001). A colorimetric *in vitro* drug sensitivity assay for *Plasmodium falciparum* based on a highly sensitive double-site lactated hydrogenase antigen-capture enzyme-linked immunosorbent assay. . *American Journal Tropical Medicine and Hygiene.,* 64, 233-241.

Duraisingh, M.T., Jones, P., Sambou, I., Seidlein, L., Pinder, M., Warhurst, D.C., (1999). Inoculum effect leads to overestimation of *in vitro* resistance for artemisinin derivatives and standard an- timalarials: a Gambian field study. *Parasitology.* 119*:* 435–440

Echezarreta, G., Yusuf, O. G., Ademowo, and S. M., Ladipo. (1997). *Sangre*. 42: 369-375.

Edwards, G., Ward, S., Breckenridge, A. (1992). Interaction of arteether with the red blood cell *in vitro* and its possible importance in the interpretation of plasma concentrations *in vivo*. *Journal of Pharmacy and Pharmacology.* 44: 280-281.

Elabbadi, N., Ancelin, M., and Vial, H. (1992). Use of radioactive ethanolamine incorporation into phospholipids to assess *in vitro* antimalarial activity by the semiautomated microdilution technique. *Antimicrobial Agents Chemotherapy.*, 36, 50-55.

Electoral Commission of kenya. (E.C.K), (1999). Registration centres by electoral area and constituency.

Ellis, M. F., David, L. S., Wendy, P. O'Meara, and Eleanor, M. R. (2008). Strain theory of malaria: the first 50 years. .*Advanced parasitology.* 66; 1-46

Eyase, F.L., Akala, H.M., Ingasia, L., Cheruiyot, A., Omondi, A., Okudo, C., Juma, D.,Yeda, R., Andagalu, B., Wanja, E., Kamau, E., Schnabel, D., Bulimo, W., Waters, N.C., Walsh, D.S., Johnson, J.D., (2013). The role of Pfmdr1 and Pfcrt in changing chloroquine, amodiaquine, mefloquine and lumefantrine susceptibility in western-Kenya *P. falciparum* samples during 2008-2011. *PLoS One.* 8, 64299.

Evavold, B.D. and Allen, P.M., (1991). *Science*, 252: 1308-1310.

Falade,, C. O., A. O., Ogundele, B. O., Yusuf, O. G., Ademowo, and S. M., Ladipo. (2008). High efficacy of two artemisinin-based combinations (artemether-lumefantrine and artesunate plus amodiaquine) for acute uncomplicated malaria in Ibadan, Nigeria. *Tropical Medicine and International Health.* 13:635–643.

Fall, B., Diawara, S., Sow, K., Baret, E., Diatta, B., Fall, K.B., Mbaye, P.S., Fall, F., Diémé, Y., Rogier C, Wade B, Bercion R, Pradines B. (2011). *Ex vivo* susceptibility of *Plasmodium falciparum* isolates from Dakar, Senegal, to seven standard anti-malarial drugs. *Malaria Journal.* 10:310.

Ferdig, M.T., Su, X-z, (2000). Microsatellite markers and genetic mapping in *Plasmodium falciparum. Parasitology Today 16:* 307–312.

Fisher, A., Laing, J., and Stoeckel, J. (1998). Guidelines for overcoming design problems in family planning operations research. In: Foreit. Family planning operations research. . *New York The Population Council. programme*, 345-555.

Florens, L., Zangwill, M. and Ginsburg, H . (2002). A proteomic view of the *Plasmodium falciparum* life cycle. *Nature.* 419,(6906): 520–526.

Fogh, S., Jepsen, S., and Efferson. (1979). Chloroquine-resistant *Plasmodium falciparum* in Kenya. *Transactions of the Royal Society of Tropical Medicine and Hygiene., 73,* 228-229.

Forum of European Societies of Clinical Chemistry (FESCC). (2006). The quality of diagnostic samples. Available at: http://www.diagnosticsample.com/.

Foster, S., and Phillips, M. (1998). Economics and its contribution to the fight against malaria. *Annals of Tropical Medicine and Parasitology, 92*: 391-398.

Freese, J. A., Rossouw, E.J., Gouws, E., Sharp, B.L., and Martins, M.B. (1993), *In vitro* sensitivity of southern African isolates of *Plasmodium falciparum* to halofantine. *Annals of Tropical Medicine and Parasitology*, 87:235-239

Freshney, R. I., edition. (1992) Animal Cell Culture: A Practical Approach. 1. 2nd edition New York: Oxford University Press, 1992: 54

Freshney, R. Ian. (1994). Culture of Animal Cells: A Manual of Basic 3. Technique. 3rd edition. New York: Wiley-Liss. 80-84.

Freshney, R.I. (2005). Culture of animal cells: A manual of basic technique. 5th edition. New York: Wiley

Freshney, R.I. (2006). Basic principles of cell culture. Hoboken: John Wiley and Sons.

Gardner, M.J, *et al.* (2002).Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature.* 419,(6906): 498–511.

Geary, T. G., Divo, A. A., Jensen, J. B., (1983). An *in vitro* assay system for the identificationof potential antimalarial drugs. *Journal of parasitology*, 69: 577–583.

Geary, T.G., Divo, A.D., Jensen, J.B., Zangwill, M. and Ginsburg, H. (1990). Kinetic modelling of the response of *Plasmodium falciparum* to chloroquine and its experimental testing *in vitro*. *Biochemical Pharmacology* 40: 685-691.

Geissler, P. W., Nokes, K., Prince, R. J., Odhiambo, R. A., Aagaard, H. J., and Ouma, J. H., (2000). Children and medicines: Selftreatment of common illnesses among Luo school children in western Kenya. *Social Science and Medicine.* 50: 1771-1783.

German, P. I., Aweeka, F. T. (2008). Clinical pharmacology of artemisinin-based combination therapies. *Clinical Pharmacokinetics*. 47:91–102.

Glik, D.C., *et al*. (1999) Malaria treatment practices among mothers in Guinea. *Journal of health and social behavior*. 30: 421-435.

Gluzman, I.Y., Schlesinger, P.H. and Krogstad, D.J. (1987). Inoculum effect with chloroquine and *Plasmodium falciparum*. *Antimicrobial Agents and Chemotherapy* 31; 32-36.

Goossens, W., Van, Duppen, V., Verwilghen, R.L. (1991). K2 or K3EDTA: the anticoagulant of choice in routine haematology? *Clinical and Laboratory Haematology*. 13:291–5.

Greenwood, B.M., and Armstrong, J.R.M. (1991). Comparison of two simple methods for determining malaria parasite density. *Transactions of the Royal Society of Tropical Medicine and Hygiene.* 85: 186-188

Guerin, P. J., Olliaro, P., Nosten, F., Druilhe, P., Laxminarayan, R., Binka, F., and White, N. J. (2002). Malaria: current status of control, diagnosis, treatment, and a proposed agenda for research and development. *Lancet Infectious Disease., 2,* 564-573.

Guo, X. B., L. C. Fu, Y. X. Fu, B. S. Qian, and G. Q. Li. (1993). Randomized comparison of the treatment of falciparum malaria with dihydroartemisinin and piperaquine. *National Medical Journal of China.* 73: 602–604

Halidou, T. Léa, N. Louis, A .N.,Isidore, Y. ,Moussa, L.,Innocent V.,Hermann, S.,Hervé, K.,Tinga, R. G.,Adama, K.,Jean B. O.,Petronella, F. M., Henk S. and Umberto, D.A.(2014). *Ex vivo* anti-malarial drugs sensitivity profile of *Plasmodium falciparum* field isolates from BurkinaFaso five years after the national policy change. *Malaria Journal* 13:207

Hamel, M. J., Odhacha, A., Roberts, J. M. and Deming, M.S. (2001). Malaria control in Bungoma District, Kenya: a survey of home treatment of children with fever, bednet use and attendance at antenatal clinics. *Bulletin of the World Health Organization.* 79**:** 1014-1023

Hao, M., Jia, D., Li, Q., (2013). *In vitro* sensitivities of *Plasmodium falciparum* isolates from the China- Myanmar border to piperaquine and association with polymorphisms in candidate genes. *Antimicrobial Agents Chemotherapy.* 57:1723–9.

Happi, C. T., Gbotosho, G. O., Sowunmi, A., Falade, C. O., Akinboye, D. O., Oladepo, O., (2004). Malaria diagnosis: false negative Parasight-F tests in falciparum malaria patients in Nigeria. *African Journal of Medicine and Medical Sciences, 33,* 15-18.

Hastings, I. M., & Alessandro, U. D. (2000). Modelling a predictable disaster: the rise and spread of drug-resistant malaria. *Parasitology Today, 16,* 340-347.

Hayton, K., and Su, X. Z. (2004). Genetic and biochemical aspects of drug resistance in malaria parasites. *Current Drug Targets Infectious Disorders,* 4, 1-10.

Hyde, J. E. (2002). Mechanisms of resistance of *Plasmodium falciparum* to antimalarial drugs. *Microbes and Infection.* 4: 165-174.

Issaka, M. Ibrahim Arzika, Julia Guillebaud, Abani Maazou, Sabine Specht, Halima Zamanka, Thierry Fandeur and Adamou Salissou. (2013). *Ex Vivo* responses of *Plasmodium falciparum* Clinical Isolates to Conventional and New Antimalarial Drugs in Niger. *Antimicrobial Agents and Chemotherapy*. 57; 7 3415–3419.

Jafari S, Le Bras J, Bouchaud O, Durand R. (2004). *Plasmodium falciparum* clonal population dynamics during malaria treatment. *Journal of Infectious Disease.* 189:195–203.

Jane, R. Z., Eve, M. L., Trenton, K. R., Allen, W. H., John E., Ben 0. W., Beverly M., Ellen, P., and Carlos, C., (1996). Childhood mortality during and after hospitalization in western Kenya: effect of malaria treatment regimens. *American Journal of tropical Medicine and hygiene.* 55, (6): 655-660.

Johnson, J. D., Dennull, R. A., Gerena, L., Lopez-Sanchez M., Roncal, N. E., Waters, N. C., (2007). Assessment and continued validation of the malaria SYBR green I-based fluorescence assay for use in malaria drug screening. *Antimicrobial Agents Chemotherapy 51:* 1926 – 1933.

Jorgensen, J. H., and M. J. Ferraro. (2000). Antimicrobial susceptibility testing: special needs for fastidious organisms and difficult-to-detect resistance mechanisms. *Clinical Infectious Disease* 30:799–808.

Kaddouri, H., A. Djimde, S. Dama, A. Kodio, M. Tekete, V. Hubert, A. Kone, H. Maiga, O. Yattara, B. Fofana, B. Sidibe, C. P. Sangare, O. Doumbo, and J. Le Bras. (2008).

Baseline *in vitro* efficacy of ACT component drugs on *Plasmodium falciparum* clinical isolates from Mali. *International Journal Parasitology*. 38:791–798.

Kaddouri, H., Nakache, S., Houze, S., Mentre, F., and Le Bras, J. (2006). Assessment of the drug susceptibility of *Plasmodium falciparum* clinical isolates from Africa by using a Plasmodium lactate dehydrogenase immuno-detection assay and an inhibitory maximum effect model for precise mea-surement of the 50-percent inhibitory concentration. *Antimicrobial Agents Chemotherapy, 50,* 3343-3349.

Kamya, M. R., Yeka, A., Bukirwa, H., (2007). Artemether-lumefantrine versus dihydroartemisinin- piperaquine for treatment of malaria: a randomized trial. *PLoS Clinical Trials* 2:e20.

Kamya, M. R., Yeka, A., Burkirwa, H., Lugemwa, M., Rwakimari, J. B., Staedke, S. G., Talisuna, A. O, Greenhouse, B., Nosten, F., Rosenthal, P. J., Wabwire-Mangen, F., Dorsey, G. (2007). Artemether- lumefantrine versus dihydroartemisinin piperaquine for treatment of malaria: a randomized trial. *Plos Clinical Trials*. 2:20

Karle, J.M., Karle, I.L., Gerena, L., Milhous, W.K. (1992). Stereo chemical evaluation of the relative activities of the cinchona alkaloids against. *Plasmodium falciparum. Antimicrobial Agents Chemotherapy*. 36: 1538-1544.

Karmiol S. (2000) Development of serum free media. In: Master JRW, editor. Animal Cell culture, 3rd edition. Oxford: Oxford University Press.

Kaseje D.C.O *et al.* (2007) Usage of community based chloroquine treatment for malaria in Saradidi, Kenya. *Annals of tropical medicine and parasitology*, 81:111-115.

Kenya Ministry of Health, (2013). "Kenya Malaria Fact Sheet." Nairobi.

Kenya Ministry of Health, (2014). "Kenya Malaria Fact Sheet." Nairobi.

Kenya Ministry of Health, (2015). "Kenya Malaria Fact Sheet." Nairobi.

Kenya Ministry of Public Health and Sanitation , M. P. H. S. (2011). "Kenya Malaria Fact Sheet." Nairobi.

Kenya Ministry of Public Health and Sanitation, M. P. H. S. (2012). National Malaria Strategy 2009–2017. Nairobi.

Khan B, Omar S, Kanyara JN, Warren-Perry M, Nyalwidhe J, Peterson DS, Wellems T, Kaniaru S, Gitonga J, Mulaa FJ, Koech DK, 1997. Antifolate drug resistance and point mutations in *Plasmodium falciparum* in Kenya. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. 91*:* 456–460.

Kilama, W. L. (2001). The malaria burden and the need for research and capacity strengthening in Africa. *American Journal of Tropical medicine and hygiene*, 1, 64.

Kobbe, R., Neuhoff, R., Marks, F., Adjei, S., Langefeld, I., von Reden, C., Adjei, O., Meyer, C.G., May, J. (2006). Seasonal variation and high multiplicity of first *Plasmodium falciparum* infections in children from a holoendemic area in Ghana, West Africa. *Tropical Medicine International Health 11:* 613–619.

Kobbe, R., P. Klein, S. Adjei, S. Amemasor, W. N. Thompson, H. Heide- mann, M. V. Nielsen, J. Vohwinkel, B. Hogan, B. Kreuels, M. Buhrlen, W. Loag, D. Ansong, and J. May. (2008). A randomized trial on effectiveness of artemether-lumefantrine versus artesunate plus amodiaquine for unsuper- vised treatment of uncomplicated *Plasmodium falciparum* malaria in Gha- naian children. *Malaria Journal.* 7:261

Kokwaro, G., L. Mwai, and A. Nzila. (2007). Artemether/lumefantrine in the treatment of uncomplicated falciparum malaria. *Expert Opinion on Pharmacotherapy.* 8:75–94

Kosaisavee, V., Suwanarusk, R., Nosten, F., Kyle, D. E., Barrends, M., Jones, J., Lek-Uthai, u. (2006). *Plasmodium vivax*: isotopic, PicoGreen, and microscopic assays for measuring

chloroquine sensitivity in fresh and cryopreserved isolates. *Experimental Parasitology, 114*, 34-39.

Kouri, T., Siloaho, M., Pohjavaara, S., Koskinen, P., Malminiemi, O., PohjaNylander, P. (2005). Preanalytical factors and measurement uncertainty. *Scandinavian Journal of Clinical and laboratory Investigation.* 65:463–76

Lan, C. X., X. Lin, Z. S. Huang, Y. S. Chen, and R. N. Guo. (1989). *In vivo* sensitivity of *Plasmodium falciparum* to piperaquine phosphate assayed in Linshui and Baisha counties, Hainan province. *Chinese Journal of Parasitology.* 7:163–165.

Lane, C., Pax, R., Bennett, J. (1987). L-glutamine: an amino acid required for maintenance of the tegumental membrane potential of *Schistosoma mansoni*. *Parasitology*. 94:233-42.

Langhorne, J., Ndungu, F.M., Sponaas, A.M., Marsh, K. (2008). Immunity to malaria: more questions than answers. *Nature Immunology.* 9, (7):725–732.

LeBras, J. (1983) *Plasmodium falciparum*: Drug sensitivity i*n vitro* of isolates before and after adaptation to continuous culture. *Experimental parasitology*. 56: 9–14.

Lee, S. A., Yeka, A., Nsobya, S. L., Dokomajilar, C., Rosenthal, P. J., Talisuna, A., Dorsey, G., (2006). Complexity of *Plasmodium falciparum* infections and antimalarial drug efficacy at 7 sites in Uganda. *Journal of Infectious Diseases. 193:* 1160–1163.

Lepe-Zuniga J.L., Zigler, J.S., Gery, I. (1987). "Toxicity of light-exposed Hepes media". *Journal of Immunological Methods.* 103 (1): 145.

Li, W. and Schlessinger, (1991). *Mol. Cell Biol.*, 11, 375b-3761.

Lim, P., Dek, D., Try, V., (2013). *Ex vivo* susceptibility of *Plasmodium falciparum* to antimalarial drugs in western, northern, and eastern Cambodia, 2011–2012: association with

molecular markers. *Antimicrobial Agents Chemotherapy.* 57:5277–83.

Lippi, G., Salvagno, G.L., Solero, G.P., Franchini, M., Guidi, G.C. (2005). Stability of blood cell counts, hematologic parameters and reticulocytes indexes on the Advia A120 hematologic analyzer. *Journal of Laboratory and Clinical Methods*. 146:333–40.

Liu, S., J. Mu, H. Jiang, and X.-Z. Su. (2008). Effects of *Plasmodium falciparum* mixed infections on *in vitro* antimalarial drug tests and genotyping. *American Journal of Tropical Medicine and Hygiene.* 79:178–184

Lorenzetti, A., P. A. Fornazari, A. C. Bonini-Domingos, R. D. S. R. Penhal- bel, É . Fugikaha, C. R. Bonini-Domingos, V. D. Fraga, L. M. Conceiçã̃o, A. R. B. Rossit, C. E. Cavasini, V. S. C. D. Couto, and R. L. D. Machado**.** (2008). Mixed *Plasmodium faciparum* infections and its clinical implications in four areas of the Brazilian Amazon region. *Acta Tropica*. 107:8–12.

Louis, J.R., Hengy, C., Louis, F.J., Gazin, F., Jamoou, R., Gardon, J., Fadat, G., Trebucq, A., (1992). Proposals for a new therapeutic strategyfor simple *Plasmodium falciparum* malaria attacks in Cameroon. *Tropical Medicine Parasitology.* 43: 118-120.

Luxemburger, C., N. J. White, F. ter Kuile, H. M. Singh, I. Allier-Frachon, M. Ohn, T. Chongsuphajaisiddhi, and F. Nosten. (1996). The epidemiology of malaria in a Karen population on the western border of Thailand. *Transactions of the Royal Society of Tropical Medicine and Hygiene.* 90:105–111.

Mackinnon, M.J., Read, A.F. (2009). Selection for high and low virulence in the malaria parasite *Plasmodium chabaudi*. *Proceedings of the Royal Society of London.* 66:741–748.

Mahaderan, L.C., Brockman, A. Jaidee, S. Nair, D. Sudimack, T. and Pongvongsa, S. (1991). *Cell*. 65: 775-783.

Mahmoud, B. M., Ali, H. M., Homeida, M. M., Bennett, J. L. (1994). Significant reduction in chloroquine biolavailability following coadministration with the Sudanese beverages Aradaib, Karkadi and Lemon. *Journal of Antimicrobial Chemotherapy*. 33: 1005-1009.

Makler, M. T., Ries, J. M., Williams, J. A., Bancroft, J. E., Piper, R. C., Gibbins, B. L., and Hinrichs, D. J. (1993). Parasite lactate dehydrogenase as an assay for *Plasmodium falciparum* drug sensitivity. . *American Journal of Tropical medicine and hygiene, 48*, 739-741.

Mariama Issaka, Ibrahim Arzika, Julia Guillebaud, Abani Maazou, Sabine Specht, Halima Zamanka, Thierry Fandeur and Adamou Salissou.(2013). *Ex Vivo* Responses of *Plasmodium falciparum* Clinical Isolates to Conventional and New Antimalarial Drugs in Niger. *Antimicrobial Agents and Chemotherapy*. 57; 7 3415–3419.

Marsh, K. (1998). Malaria disaster in Africa. *Lancet. 352*, 924-925.

Maude, R. J., Pontavornpinyo, W., Saralamba, S., Aguas, R., Yeung, S., Dondorp, A. M., Day N. P. J., White, N. J., White, L. J. (2009). The last man standing is the most resistant: eliminating artemisinin-resistant malaria in Cambodia. *Malarial Journal*. 8:31

Mayxay, M., M. Barends, A. Brockman, A. Jaidee, S. Nair, D. Sudimack, T. Pongvongsa, S. Phompida, R. Phetsouvanh, T. Anderson, N. J. White, and P. N. Newton. (2007). *In vitro* antimalarial drug susceptibility and *pfcrt* mutation among fresh *Plasmodium falciparum* isolates from the Lao PDR (Laos). *Americal Journal of Tropical Medicine and Hygiene.* 76:245–250

Mayxay, M., S. Pukrittayakamee, P. N. Newton, and N. J. White. (2004). Mixed-species malaria infections in humans. *Trends Parasitology.* 20:233–240

Mbaisi, A., Liyala, P., Eyase, F., Achilla, R., Akala , H., Wangui, J., Mwangi, J., Osuna, F., Alam, U., Smoak, B.L., Davis, J.M., Kyle, D.E., Coldren, R.L., Mason, C., Waters

N.C., (2004). Drug susceptibility and genetic evaluation of *Plasmodium falciparum* isolates obtained in four distinct geographical regions of Kenya. *Antimicrobial Agents Chemotherapy.* 48*:* 3598–3601.

Mc Elnay, J. C., Mukhtar, H. A., D'arcy, P. F., Temple, D. J. (1982). *In vitro* experiments on chloroquine and pyrimethamine absorption in the presence of antiacid constituents on Kaolin. *American Journal of Tropical Medicine and Hygiene. 85*: 153-158

Melissa, D. Conrad, Norbert, LeClair, Emmanuel, Arinaitwe, Humphrey Wanzira, Abel Kakuru, Victor Bigira,Mary Muhindo, Moses R. Kamya, Jordan W. Tappero, Bryan Greenhouse, Grant Dorsey, and Philip J. Rosenthal, (2014) Comparative Impacts Over 5 Years of Artemisinin-Based Combination Therapies on Plasmodium falciparum Polymorphisms That Modulate Drug Sensitivity in Ugandan Children. *Journal of Infectious Diseases* 210:344–53.

Mendelson, J., Caviles, A. and Castagnola, J. (2001). Culture of human lymphocytes in serum-free medium. In: Barnes DW, Sirbasku DA, Sato GH, editors. Methods for serum-free culture of neuronal and lymphoid cells. New York: Liss.

Meshnick, S.R., Thomas, A., Ranz, A., Xu CM, Pan H.Z., (1991). Artemisinin (qinghaosu): the role of intracellular hemin in its mechanism of antimalarial action. *Molecular and Biochemical Parasitology.* 49: 181-190.

Mordmuller, B., and Kremsner, P. G. (2006). Malarial parasites versus antimalarials: never-ending rumble in the jungle. *Current Molecular Medicine. 6*: 247-251.

MS - Ministerio de Salud, Colombia (1999). Guía de Atención clínica para el diagnóstico y tratamiento de la malaria. Ministerio de Sa- lud - Dirección General de Promoción y Prevención, bogotá DC, Colombia. *Antimicrobial Agents Chemotherapy.*, 31:3575-3577.

Murray, C.J., Rosenfeld, L.C., Lim, S.S., (2014). Global malaria mortality between 1980 and 2013: a systematic analysis. *Lancet* 379:413.

Mwai L, Ochong E, Abdirahman A, Kiara SM, Ward S, Kokwaro G, Sasi P, Marsh K., Borrmann S, Mackinnon M, Nzila A, (2009). Chloroquine resistance before and after its withdrawal in Kenya. *Malaria Journal. 8:*106.

Mwai, L., Kiara, S.M., Abdirahman, A., (2009). *In vitro* activities of piperaquine, lumefantrine, and dihydroartemisinin in Kenyan *Plasmodium falciparum* isolates and polymorphisms in pfcrt and pfmdr1. *Antimicrobial Agents Chemotherapy.* 53:5069–73.

Myint, H. Y., Ashley, E. A., Day, N. P. J., Nosten, F., White, N. J. (2007). Efficacy and safety of dihydroartemisinin-piperaquine. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. 101:858-866.

Myint, P. T., Shwe, T., (1987). A controlled clinical trial of artemether (qinghaosu derivative) versus quinine in complicated and severe falciparum malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. 81: 559-561.

Nagle, S. (1999). Heat stable chemically defined medium for growth of animal cells in suspension. *Applied Microbiology*. 16:53-5.

Narayanan, S. (1993). Effect of anticoagulants used for blood collection on laboratory tests. *Proceedings JCLA* , 7:1–10.

Nevill, C.G., Verhoeff, F.H., Munafu, C.G., Ten Hove, W.R., VanderKaay, H.I., Were, J.B.O, (1994). A comparison of amodiaquine and chloroquine in the treatment therapy of falciparum malana in Kenya. *East African Medical Journal.* 71: 167-170.

Ng'Guessan, R., Corbel, V., Akogbeto, M., and Rowland, M. (2007).Reduced efficacy of insecticide-treated nets and indoor residual spraying for malaria control in pyrethroid resistance area, Benin. *Emerging Infectious Diseases.* 13,(2):199–206.

Noedl, H., Bronnert, J., Yingyuen, K., Attlmayr, B., Kollaritsch, H., & Fukuda, M. (2005). Simple histidine-rich protein 2 double-site sandwich enzyme linked immunosorbent assay for use in malaria drug sensitivity testing. *Antimicrobial Agents Chemotherapy.*, *49*:3575-3577.

Noedl, H., Se, Y., Sriwichai, S., Schaecher, K., Teja-Isavadharm, P., Smith, B., Rutvisuttinunt, W, Bethell, D., Surasri, S., Fukuda, M. M., Socheat, D., Chan Thap, L. (2010). Artemisinin resistance in Cambodia: a clinical trial designed to addressan emerging problem in Southeast Asia. *Clinical Infectious Disease.* 51:82–89.

Noedl, H., Wernsdorfer, W. H., Miller, R. S., & Wongsrichanalai, C. (2002). Histidine-rich protein II: a novel approach to malaria drug sensitivity testing. *Antimicrobial Agents Chemotherapy. 46*: 1658-1664.

Noedl, H., Wongsrichanalai, C., & W.H, W. (2003). Malaria drug-sensitivity testing: new assays, new perspectives. *Trends Parasitology.* 19: 175-181.

Nosten, F, and White, N. J. (2007). Artemisinin-based combination treatment of falciparum malaria. *American Journal of Tropical Medicine and Hygiene.*; 77:181–92.

Nsobya, S. L., Kiggundu, M., Nanyunja, S., Joloba, M., Greenhouse, B., Rosenthal, P.J. (2010). *In vitro* sensitivities of *Plasmodium falciparum* to different antimalarial drugs in Uganda. *Antimicrobial Agents Chemotherapy.* 54:1200–6.

Ofulla, A. O., Orago, A. S., Githure, J. I., Burans, J. P., Aleman, G. M., Johnson, A. J., & Martin, S. K. (1994). Determination of fifty percent inhibitory concentrations (IC_{50}) of

antimalarial drugs against *Plasmodium falciparum* parasites in a serum-free medium. *51*, 214-218.

OPS-OMS - Organización Panamericana de la Salud, Organización Mundial de la Salud (1998). Evaluación de la eficacia terapéutica de los medicamentos para el tratamiento del paludismo por *Plas- modium falciparum* sin complicaciones en las Américas. Washington. Available from: paho.org/spanish/HCP/HCT/doc452.pdf

Osorio, L.E., Pérez, L.P., González, I.J. (2007). Assessment of the efficacy of antimalarial drugs in Tarapacá, in the Colombian Amazon basin. *Biomedical. 27*: 133-140.

Ouellette, M. (2001). Biochemical and molecular mechanisms of drug resistance in parasites. *Tropical Medicine and International Health, 6*, 874-876.

Pan, H.Z, Lin, F.B, Zhang, Z.N, (1989). Effect of sodium artesunate on malaria infected human erythrocytes. *Proceedings of the Chinese Academy of Medical Sciences Peking Union Medical CoIlege Journal*. 4: 181-185.

Parola, P., B. Pradines, F. Simon, M. P. Carlotti, P. Minodier, M. P. Ran- jeva, S. Badiaga, L. Bertaux, J. Delmont, M. Morillon, R. Silai, P. Brouqui, and D. Parzy. (2007). Antimalarial drug susceptibility and point mutations associated with drug resistance in 248 *Plasmodium falciparum* isolates im- ported from Comoros to Marseille, France in 2004–2006. *American Journal of Tropical Medicine and Hygiene*. 77:431–437.

Pascual, A., Parola, P., Benoit-Vical, F., Simon, F., Malvy, D., Picot, S., Delaunay, P., Basset, D., Maubon, D., Faugère, B., Ménard, G., Bourgeois, N., Oeuvray, C., Didillon, E., Rogier, C. P. B. (2012). *Ex vivo* activity of the ACT new components pyronaridine and piperaquine in comparison with conventional ACT drugs against isolates of *Plasmodium falciparum*. *Malarial Journal*. 11:9.

Pasieka, A., and Morgan, J. (1959). Glutamine metabolism of normal and malignant cells cultivated in synthetic media. *Nature.* 183:1201-2.

Payne, D., Wernsdorfer, W. H. (1989). Development of a blood culture medium and a standard *in vitro* microtest for field-testing the response of *Plasmodium falciparum* to antifolate antimalarials. *Bulletin of the World Health Organization,* 67: 59–64.

pdf, 371;5.

Perrotta, G., Roberts, L., Glazier, J., Schumacher, H.R. (1998). Use of sodium citrate anticoagulant for routine haematological analysis on the CD4000: an opportunity to enhance efficiency in the clinical laboratory. *International Journal of Laboratory Hematology*. 4:156–62.

Petersen, E. (1990). *In vitro* and *in vivo* susceptibility of Plasmodium falciparum isolates from Liberia to pyrimethamine, cycloguanil and chlorcycloguanil. *Annals of Tropical Medicine and Parasitology,* 84; 563–571.

Peterson, D. S., Milhous, W. K., & Wellems, T. E. (1990). Molecular basis of differential resistance to cycloguanil and pyrimethamine in *Plasmodium falciparum* malaria. *Proceedings of the National Academy of Sciences. 87*:3018-3022.

Plowe, C. V., Cortese, J. F., Djimde, A., Nwanyanwu, O. C., Watkins, W. M., Winstanley, P. A., O.K, D. (1997). Mutations in *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthase and epidemiologic patterns of pyrimethamine-sulfadoxine use and resistance. *Journal of Infectious Diseases. 176*: 1590-1596.

Postigo, M., A. Mendoza-León, and H. A. P´erez. (1998). Malaria diagnosis by the polymerase chain reaction: a field study in south-eastern Venezuela. *Transactions of the Royal Society of Tropical Medicine Hygiene.* 92:509–511

Pradines, B., C. Rogier, T. Fusai, A. Tall, J. F., Trape, and J. C. Doury. (1998). *In vitro* activity

of artemether against African isolates (Senegal) of *Plasmodium falciparum* in comparison with standard antimalarial drugs. *American Journal of Tropical Medicine and Hygiene* 58:354–357

Pradines, B., Mamfoumbi, M. M., Parzy, D., M.O, M., Lebeau, C., Mbina, J. R. M., Kombila, M. (1999). *In vitro* susceptibility of African isolates of *Plasmodium falciparum* from Gabon to pyronaridine. *American Journal of Tropical medicine and hygiene*, 60: 105-108.

Pradines, B., P. Hovette, T. Fusai, H. L. Atanda, E. Baret, P. Cheval, J. Mosnier, A. Callec, J. Cren, R. Amalvict, J. P. Gardair, and C. Rogier. (2006). Prevalence of in vitro resistance to eleven standard or new antimalarial drugs among *Plasmodium falciparum* isolates from Pointe-Noire, Republic of the Congo. *Journal of Clinical Microbiology*. 44:2404–2408.

Pradines, B., P. Hovette, T. Fusai, H. L. Atanda, E. Baret, P. Cheval, J. Mosnier, A. Callec, J. Cren, R. Amalvict, J. P. Gardair, and C. Rogier. (2006). Prevalence of *in vitro* resistance to eleven standard or new antimalarial drugs among *Plasmodium falciparum* isolates from Pointe-Noire, Republic of the Congo. *Journal of Clinical Microbiology* 44:2404–2408.

Pradines, B., A. Tall, T. Fusai, A. Spiegel, R. Hienne, C. Rogier, J. F. Trape, J. Le Bras, and D. Parzy. (1999). *In vitro* activities of benflumetol against 158 Senegalese isolates of *Plasmodium falciparum* in comparison with those of standard antimalarial drugs. *Antimicrobial Agents Chemotherapy*. 43:418–420.

Price, R. N., Hasugian, A. R., Ratcliff, A., Siswantoro, H., Purba, H. L. E., Kenangalem, E, Lindegardh, N., Penttinen, P., Laihad, F., Ebsworth, E. P., Anstey, N. M., Tjitra, E. (2007). Clinical and pharmacological determinants of the therapeutic response to

dihydroartemisinin- piperaquine for drug- resistant malaria. *Antimicrobial Agents Chemotherapy.* 51:4090-4097.

Quellette, M. (2001). Biochemical and molecular mechanisms of drug resistance in parasites. *Tropical Medicine and International Health. 6*: 874-876.

Rason MA, Randriantsoa T, Andrianantenaina H, Ratsimbasoa A, Menard D, (2008). Performance and reliability of the SYBR Green I based assay for the routine monitoring of susceptibility of *Plasmodium falciparum* clinical isolates. *Transactions of the Royal Society of Tropical Medicne and Hygiene.* 102*:* 346–351.

Rathod, P. K., McErlean, T., Lee, P. C. (1997). Variations in frequencies of drug resistance in *Plasmodium falciparum*. *Proceedings of the National Academy of Sciences of the United States of America* 94: 9389–9393

Raynal A.L., (2005). Use of over the counter medications in rural Matabeleland, Zimbabwe: the case for upgrading the dispensing skills of rural shopkeepers. *Central African Journal of medicine.* 31: 92-97.

Reardon, D.M., Warner, B., Trowbridge, E.A. (1991). EDTA, the traditional anticoagulant of haematology: with increased automation is it time for review? *Medical Laboratory Science.* 48:72–5

Reardon, D.M., Warner, B., Trowbridge, E.A. (2003). EDTA, the traditional anticoagulant of haematology: with increased automation is it time for review? *Medical Laboratory Science*. 48:72–5.

Rengarajan, K., Cristol, S., Mehta, M., and Nickerson, J. (2002). Quantifying DNA concentrations using fluorometry: a comparison of fluorophores. *Molecular Vision.* 8: 416-421.

Richie, T. L. (1988). Interactions between malarial parasites infecting the same vertebrate host. *Parasitology*. 96: 607–639

Rieckmann, K. H., Campbell, G. H., Sax, L. J., and Ema, J. E. (1978). Drug sensitivity of *Plasmodium falciparum*: an in-vitro microtechnique. *The Lancet.* 311, (8054): 22-23.

Ringwald, P., Basco, L. K. (1999). Comparison of *in vivo* and *in vitro* tests of resistance in patients treated with chloroquine in Yaoundé, Cameroon. *Bulletin of the World Health Organization 77*: 34-43.

Ritchie, G. Y., Mungthin, M., Green, J. E., Bray, P. G., Hawley, S. R. and ward, S. A. (1996). *In vitro* selection of halofantrine resistance in *Plasmodium falciparum* is not associated with increased expression of Pgh1.*Molecular and Biochemical Parasitology*. 83: 35-46

Romeo, C., and Seed, B. (1991). *Cell*. 64, 1037-1046.

Romero, A., Matos, C., Gonzalez, M. M., Nuñez, N., Bermudez, L., De Castro, G. (1993). Alteraciones de la mucosa gastrica en malaria aguda. *Genetics.. 47*: 123-128.

Rooney, W., Thimasarn, K., (1991). Mefloquine plus sulfadoxine and pyrimethamine (letter). *Lancet.* 338: 352.

Ruebush, T. K., Kern, M. K., Campbell, C. C. and Oloo, A. J. (1995). Self treatment of malaria in a rural area of western Kenya. *Bulletin of the World Health Organization.* 73, 229-236.

Ruebush, T. K., Marquiño, W., Zegarra, J., Neyra, D., Villaroel, R., Avila, J. C., Díaz, C., Beltrán, E. (2003). Practical aspects of *in vivo* antimalarial drug efficacy testing in the Americas. *American Journal of Tropical Medicine and Hygiene. 68*: 391-397.

Sachs, J., and Malaney, P. (2002). The economic and social burden of malaria. *Nature., 415*, 680-685.

Sammy, K., Nicky, O., Bartholomew, N. O., and Bernard, K. L. (2013). Choice and Sources of Antimalarial Drugs Used for Self-medication in Kisumu, Western Kenya. *African Journal of Pharmacology and Therapeutics, 2*(4), 124-129.

Sauerborn R *et al*. (2009). Low utilization of community health workers: results from a household interview survey in Burkina Faso. *Social science and medicine.* 29: 1163-1174.

Schapira, A., Bygbjerg, C. I., Jepsen, S. Flachs, H. and Michael, W. B., (1986). The Susceptibility of *Plasmodium Falciparum* to Sulfadoxine and Pyrimethamine: Correlation of *in vivo* and *in vitro* Results. *American Journal of Tropical Medicine and Hygiene. 35:239-245*

Scherf, A., Lopez-Rubio, J.J., Riviere, L. (2008). Antigenic variation in *Plasmodium falciparum*. *Annual Review of Microbiology.* 62:445–470.

Shi, S.R., Kelly J. X., Wilairat P, Riscoe, M. (1993). *Journal of Histochemistry and Cytochemistry*. 41: 1599-1604.

Shipman, C. (1969). Evaluation of 4-(2-hydroxyethyl)-1-piperazineëthanesulfonic acid (HEPES) as a tissue culture buffer. *Proceedings of the Society for Experimental Biology and Medicine.*130:305-10.

Shretta, R., Omumbo, J., Rapuoda, B., & Snow, R. W. (2000). Using evidence to change antimalarial drug policy in Kenya. *Tropical Medicine of International Health. 5*, 755-764.

Sibley, C.H., Barnes, K. I., Watkins, W. M., Plowe, C. V. (2008). A network to monitor antimalarial drug resistance: a plan for moving forward. *Trends Parasitology. 24*: 43-48.

Sinclair, D., Zani, B., Donegan, S., Olliaro, P., Garner, P. (2009). Artemisinin-based combination therapy for treating uncomplicated malaria. *Cochrane Database of Systematic Reviews* CD007483.

Sisowath, C, Petersen I, Veiga MI, Martensson A, Premji Z, (2009). *In vivo* selection of *Plasmodium falciparum* parasites carrying the chloroquine susceptible Pfcrt K76 allele after treatment with artemether-lumefantrine in Africa. *Journal of Infectious Diseases*. 199: 750–757.

Sisowath, C., Stromberg, J., Martensson, A., Msellem, M., Obondo, C., Bjorkman, A., & Gil, J. P. (2005). *In vivo* selection of *Plasmodium falciparum* pfmdr1 86N coding alleles by artemether-lumefantrine (Coartem). *Journal of Infectious Diseases. 191*, 1014-1017.

Skeidsvall, J., and Veland, P. (1995). Analysis of double-stranded DNA capillary electrophoresis laser-induced fluorescence detection using the monomeric dye SYBR Green I. *Analytical Biochemistry. 231*, 359-365.

Slater, A.F.G., Cerami, A., (1992). Inhibition by chloroquine of a novel haem polymerase enzyme activity in malaria trophozoiles. *Nature.* 355:167-169.

Smilkstein, M., Sriwilaijaroen, N., Kelly, J. X., Wilairat, P. and Riscoe M. (2004). Simple and inexpensive fluorescence-based technique for high-throughput *Antimalarial Agents Chemotherapy*. 48*:* 1803–1806.

Snow, R. W. (1999). Estimating mortality, morbidity and disability due to malaria among Africa's non-pregnant population. *Bulletin of the World Health Organization., 77*, 624-640.

Stoll, T., Muhlethaler, K., von Stockar, U. and Marison, I.(1996). Systematic improvement of a chemically-defined protein-free medium for hybridoma growth and monoclonal antibody production. *Journal of Biotechnology*. 45:111-23.

Trager W., Jenson J. (1978). Cultivation of malarial parasites. *Nature.* 273: 621–622

Trager, W., Jensen, **J. B.** (1976**).** Human malaria parasites in continuous culture. *Science.* 193: 836-839.

Trape, J. F, Pison G. Preziosi, M. P, (1998). Impact of chloroquine resistance on malaria mortality. *Comptes Rendus deAcademie des Sciences series 111*. 321: 689–97.

Tulpule, A., Krishanaswamy, K. (1982). Effect of food on bioavailability of chloroquine. *European Journal of Clinical Pharmacology. 23*:271-273.

Tulpule, A., Krishanaswamy, K. (1983). Effect of rice diet on chloroquine bioavailability. *Drug Nutrition Interactions 2*: 83-86.

Uszyński, W., Andersen, L. Gerena, and W. K. Milhous. (2007). *Folia Histochemica et Cytobiologica.* 45: 33-36.

V Ööpik, I Saaremets, L. Medijainen, K. Karelson, T. Janson, S. Timpmann (2003). "Effects of sodium citrate ingestion before exercise on endurance performance in well trained college runners". *British Journal of Sports Medicine.* 37, (6): 485–489.

Vandemark, P.J. and Smith, P.F. (1964). *Journal of Bacteriology*. 88: 1602-1607.

Vennerstrom, J. L., W. Y. Ellis, A. L. Ager, S. L. Andersen, L. Gerena, and W. K. Milhous. (1992). Bisquinolines. 1 N, N-bis (7-chloroquinolin-4-yl) alkanediamines with potential against chloroquine-resistant malaria. *Journal of Medicinal Chemistry* 35:2129–2134

Vitzthum, F., Geiger, G., Bisswanger, H., Brunner, H., & Bernhagen, J. (1991). A quantitative fluorescence-based microplate assay for the determinationof double-stranded DNA using SYBR Green I and standard ultraviolet trans illuminator gel imaging system. *Analytical Biochemistry, 276*, 59-64.

Vossen, M. G., Pferschy, S., Chiba, P., and Noedl, H. (2010). The SYBR Green I malaria drug sensitivity assay: performance in low parasitemia samples. . *American Journal of Tropical medicine and hygiene*, 82, 398-401.

Vulule, J. I., Beach, R. F., Atieli, F.K., McAllister, J.C., Brogdon, W.G., Roberts, J., Mwangi, R.M. and W. A.Hawley, W.A. (1999). Elevated oxidase and esterase levels associated with permethrin tolerance in Anopheles gambiae from Kenyan villages using permethrin-impregnated nets. *Medical and Vetinary Entomology*. 13: 239-244.

W.H.O, 2014. Status Report on Artemisinin Resistance. Available at: http://www.who.int/malaria/publications/atoz/status rep artemisinin resistance jan2014.

Walliker D., Quakyi, I.A., Wellems, T.E., McCutchan, T.F,, Szarfman, A, (1987). Genetic analysis of the human malaria parasite *Plasmodium falciparum*. *Science.* 236: 1661–1666.

Warsame ,M., Wernsdorfer, W. H. Payne, D. and Bjorkman, A. (1991). Susceptibility of *Plasmodium falciparum in vitro* to chloroquine, mefloquine, quinine and sulfadoxine/pyrimethamine in Somalia: relationships between the responses to the different drugs. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 85: 570–571.

Watsierah, C. A., Jura, W. G., Raballah, E., Kaseje, D., Abong'o, B., Ouma, C. (2011). Knowledge and behaviour as determinants of antimalarial drug use in a periurban population from malariaholoendemic region of western Kenya. *Malaria Journal. 10*, 99-105.

Watt 0, Loesuttivibool L, Shanks, G. D., Boudreau, E. F, Brown, A.E., Pavanand, K., Webster, H.K., Wechgritaya, (1992). Quinine with tetracycline for the treatment of drug-resistant

falciparum malaria in Thailand. *American Journal of Tropical Medicine and Hygiene.* 47: 108-111.

Webster, H. K., Boudreau, E. F., Pavanand, K., Yongvanitchit, K., and Pang, L. W. (1985). Antimalarial drug susceptibility testing of *Plasmodium falciparum* in Thailand using a microdilution radioisotope method. *American Journal of Tropical Medicine and Hygiene., 34*, 228-235.

Wellems, T., and Plowe, C. (2001). Chloroquine resistant malaria. *Journal of Infectious Diseases, 184*, 770-776.

Wernsdorfer W. H. (1994). Epidemiology of drug resistance in malaria. *Acta Tropica,* 56: 143–156.

Wernsdorfer, W.H., (1991). The development and spread of drug-resistant malaria. *Parasitology Today*. 7: 297-303.

Wernsdorfer, W.H., Payne, D, (1991). The dynamics of drug resistance in *Plasmodium falciparum. Pharmacology and Therapeutics.* 50: 95-121

WHO-World Health Organization (1990). Practical chemotherapy of malaria. World Health Organisation Technology. Rep Ser 805

WHO. (1990). *In vitro* Microtest (Mark II) for the assessment of response of *Plasmodium falciparum* to chloroquine, mefloquine, quinine, sulfadoxine/pyrimethamine and amodiaquine. *World Health Organization, Geneva, WHO document MAP/87.2, Rev.1.*

WHO. (1993). World malaria situation in 1993, part I. Weekly Epidemiological Record 1996. *71, 17-22*.

WHO (1996) Assessment of therapeutic efficacy for uncomplicated falciparum malaria in areas with intense transmission. Geneva, (unpublished document WHO/MAL/96.1077) available upon request from Division of Control of Tropical Diseases, World Health Organization, 1211 Geneva 27, Switzerland

WHO (2000). Expert Committee on Malaria, 20th Report. Geneva: World Health Organization Technical Report Series. *892*.

WHO (2001). *In vitro* micro-test (MARK III) for the assessment of the response of *Plasmodium falciparum* to chloroquine, mefloquine, quinine, amodiaquine, sulfadoxine/pyrimethamine and artemisinin. *World Health Organization: . Geneva.*

WHO (2003). Assessment and monitoring of antimalarial drug efficacy for the treatment of uncomplicated falciparum malaria. Geneva. Available from: whqlibdoc.who.int/ hq/2003/WHO_HTM_RbM_2003.50.pdf.

WHO, (2007). Containment of Malaria Multi-Drug Resistance on the Cambodia Thailand Border. Switzerland, Geneva: Report of an Informal Consultation Phnom Penh:23.

WHO PAR Part 4 (2010). Guidelines on Treatment of Malaria. 2nd edition.

WHO (2010). Guidelines for the treatment of malaria. Geneva, Switzerland:

WHO (2012) World Malaria Report. http://www.who.int/malaria/publications/world_malaria report 2012/report/en/

WHO (2013) World Malaria Report. http://www.who.int/malaria/publications/world_malaria report 2013/report/en/

WHO (2015) World Malaria Report. http://www.who.int/malaria/publications/world_malaria report 2015/report/en/

Wirjanata, G., Handayuni, I., Prayoga, P., Dwi Apriyanti, Chalfein, F., Steven Kho, Rintis Noviyanti, (2015). Quantification of *Plasmodium ex vivo* drug susceptibility by flow cytometry. *Malaria journal* 14:417

Yavo, W., Faye, B., Kuete, T., (2011). Multicentric assessment of the efficacy and tolerability of dihydroartemisinin-piperaquine compared to artemether-lumefantrine in the treatment of uncomplicated *Plasmodium falciparum* malaria in sub-Saharan

Africa. *Malaria Journal*. 10:198.

Yeka. A., Dorsey, G., Kamya, M. R. (2008). Artemether-lumefantrine versus dihydroartemisinin-piperaquine for treating uncomplicated malaria: a randomized trial to guide policy in Uganda. *PLoS One*; 3:e2390.

Yewhalaw, D., W. Van Bortel, L. Denis, M. Coosemans, L. Duchateau, and N. Speybroeck. (2010). First Evidence of High Knockdown Resistance Frequency in *Anopheles arabiensis* (Diptera: Culicidae) from Ethiopia. *American Journal of Tropical Medicine and Hygiene*. 83: 122–125.

Zigler, J.S., Lepe-Zuniga, J.L., Vistica, B., Gery, I. (1985). "Analysis of the cytotoxic effects of light-exposed HEPES-containing culture medium". *In Vitro Cellular and. Developmental Biology*. 21 (5): 282– 7.

Zimmerman, P. A., Mehlotra, R. K., Kasehagen, L. J. and Kazura, J. W. (2004). Why do we need to know more about mixed Plasmodium species infections in humans? *Trends in Parasitology*. 20:440–447

Zongo, I., Dorsey, G., Rouamba, N., (2007). Artemether-lumefantrine versus amodiaquine plus sulfadoxine-pyrimethamine for uncomplicated falciparum malaria in Burkina Faso: a randomised non-inferiority trial. *Lancet*; 369:491–8.

Appendix 1: Data collection instrument for demographic characteristics and parasitaemia

Serial no.	Name of the patient	Place of residence	sex	age	Parasitaemia level
1					
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3					
4					
5					
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31					
32					
33					
34					
35					
36					
37					
38 to 322					

Appendix 2: Data collection instrument for reference clones IC_{50}

	W2 clone		3D7 clone	
Serial no.	In EDTA	In TM	In EDTA	In TM
1				
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37				
38 to 322				

Appendix 3: Data collection instrument for the field isolates

Serial no.	IC_{50} for samples in EDTA	IC_{50} for samples in TM
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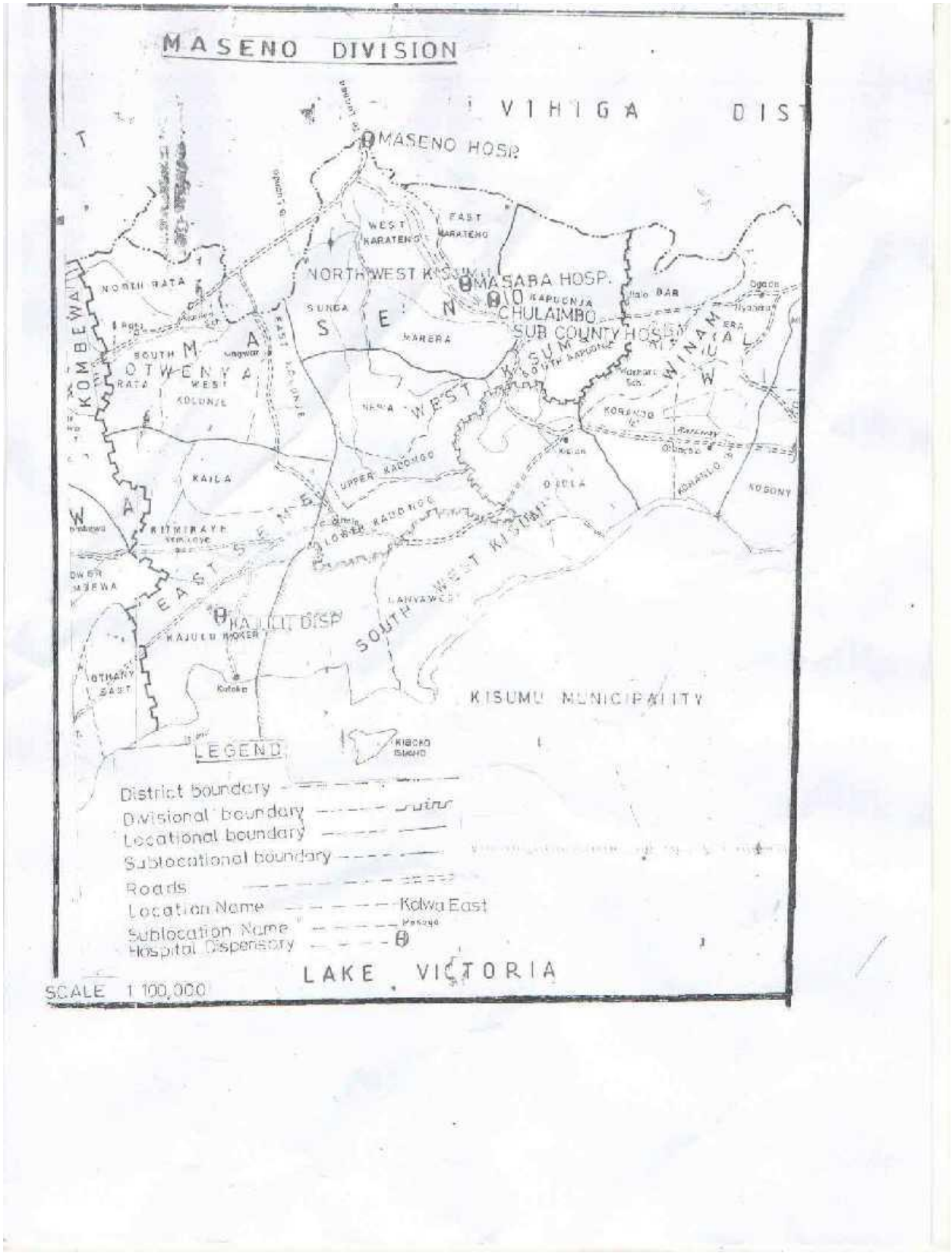
39 to 322		

Appendix 4: Map of Maseno Division



Appendix 5: Consent form

In English

I am Scolastica Chepngetich Korir and pursing a PhD in Medical Parasitology degree in Maseno University department of Biomedical Sciences & Technology. I am conducting a research on "Evaluation of a new *in-vitro* antimalarial drugs sensitivity testing technique for routine clinical use and pharmacoepidemiology at Chulaimbo Sub District Hospital in Kisumu County", as a fulfillment of the requirements for the award of the degree in Doctor of Philosophy in Medical Parasitology. I would like to recruit you to partcipate in my study as a respondent. As a respondent, your participation in the study is voluntary, and access to health care will not be dependent on participation, Moreover, you are free to withdraw at any time without having to incur any penalty. Confidentiality and privacy will be strictly safeguarded and observed. Furthemore, this research is for the purpose of my PhD study work and will not be used for any other purpose whatsoever. If you agree to partcipate in the study then append your signature.

Respondents signature/finger print ..... Date .....

Thank you in advance

Yours sincerely

Scolastica C. Korir

In Dholuo

An Scolastica Chepngetich Korir to asomo kos ma iluongo ni PhD in Medical Parasitology e Mbalariany ma Maseno, sikul mar Biomedical Sciences & Technology. Atimo nonro e gima iluongo ni "Evaluation of a new *in-vitro* antimalarial drugs sensitivity testing technique for routine clinical use and pharmacoepidemiology e Chulaimbo Sub District Hospital eyi Kisumu County", kaka achiel kuom gik ma idwaro mondo mi ayud degree mar Doctor of Philosophy in Medical Parasitology. Arwaki mondo ikonya e nonroni kaka jadwoko.

Kaka jadwoko, konyni e nonroni en kuom chiwuruok, to kendo nyalo mar yudo thieth ok biluwore gi konyni. Ewi mano, oyieni weyo nonroni e saa moro amora maonge kum moro amora ma ibiromiyi. E nonroni nyaka ibed ngat ma nyalo kano siri. Ewi mano, nonroni en mana mar somba mar PhD kendo ok bi tii kode e yo moro amora machielo. Ka iyie konyo e nonroni to akwayi ni mondo iket seyi mari piny ka.

Seyi..... Tarik .....

Erokamano

Mari

Scolastica C. Korir

Appendix 6: Ethical Approval

MINISTRY OF HEALTH

Telegrams: "MEDICAL", Kisumu
Telephone: 057-2020801/2020803/2020321
Fax: 057-2024337
E-mail: ercjootrh@gmail.com
When replying please quote

JARAMOGI OGINGA ODINGA TEACHING & REFERRAL HOSPITAL
P.O. BOX 849
KISUMU

ERC.1B/VOL.I/177

21st May, 2015

Ref: .....

Date .....

Scolastica C. Korir,
Reg. No. PG/PHD/0005/2013,
MASENO UNIVERSITY.

Dear Scolastica,

RE: FORMAL APPROVAL TO CONDUCT RESEARCH TITLED: *"EVALUATION OF A NEW IN-VITRO ANTIMALARIAL DRUGS SENSITIVITY TESTING TECHNIQUE FOR ROUTINE CLINICAL USE AND PHARMACOEPIDEMIOLOGY AT CHULAIMBO SUB-DISTRICT HOSPITAL IN KISUMU COUNTY"*

The JOOTRH ERC (ACCREDITATION NO. 01713) has reviewed your protocol and found it ethically satisfactory. You are therefore, permitted to commence your study immediately. Note that this approval is granted for a period of one year (21st May, 2015 to 22nd May, 2016). If it is necessary to proceed with this research beyond the approved period, you will be required to apply for further extension to the committee.

Also note that you will be required to notify the committee of any protocol amendment(s), serious or unexpected outcomes related to the conduct of the study or termination for any reason.

Finally, note that you will also be required to share the findings of the study in both hard and soft copies upon completion.

The JOOTRH ERC takes this opportunity to thank you for choosing the institution and wishes you the best in your endeavours.

Yours sincerely,

JOOTRH ETHICS & REVIEW COMMITTEE
P. O. Box 849 - 40100
KISUMU

FRED OUMA AKWATTA,
SECRETARY - ERC,
JOOTRH – KISUMU.

Appendix 7: Approval by Chulaimbo Sub County Hospital

MINISTRY OF HEALTH

Telegrams: "MEDICAL", Kisumu
Telephone: 057-2020801/2020803/2020821
Fax: 057-2024337
E-mail: ercjootrh@gmail.com
When replying please quote

JARAMOGI OGINGA ODINGA TEACHING & REFERRAL HOSPITAL
P.O. BOX 849
KISUMU

Ref: ERC.1B/VOL.I/177

Date: 21st May, 2015

Scolastica C. Korir,
Reg. No. PG/PHD/0005/2013,
MASENO UNIVERSITY.

Dear Scolastica,

RE: FORMAL APPROVAL TO CONDUCT RESEARCH TITLED: *"EVALUATION OF A NEW IN-VITRO ANTIMALARIAL DRUGS SENSITIVITY TESTING TECHNIQUE FOR ROUTINE CLINICAL USE AND PHARMACOEPIDEMIOLOGY AT CHULAIMBO SUB-DISTRICT HOSPITAL IN KISUMU COUNTY"*

The JOOTRH ERC (ACCREDITATION NO. 01712) has reviewed your protocol and found it ethically satisfactory. You are therefore, permitted to commence your study immediately. Note that this approval is granted for a period of one year (21st May, 2015 to 22nd May, 2016). If it is necessary to proceed with this research beyond the approved period, you will be required to apply for further extension to the committee.

Also note that you will be required to notify the committee of any protocol amendment(s), serious or unexpected outcomes related to the conduct of the study or termination for any reason.

Finally, note that you will also be required to share the findings of the study in both hard and soft copies upon completion.

The JOOTRH ERC takes this opportunity to thank you for choosing the institution and wishes you the best in your endeavours.

Yours sincerely,

JOOTRH ETHICS & REVIEW COMMITTEE
P. O. Box 849 - 40100
KISUMU

FRED OUMA AKWATTA,
SECRETARY - ERC,
JOOTRH – KISUMU.