ASSESSMENT OF *PLASMODIUM* SPECIES PREVALENCE, ANTIMALARIAL DRUG RESISTANCE GENES AND GAMETOCYTES INMALARIA INFECTIONSBETWEEN 2018 AND 2021 IN KOMBEWA SUB-COUNTY, KENYA

BY

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DECLARATION

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

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DEDICATION

I would like to dedicate this work to my husband Clement and son Ethan for their support emotionally, physically, and financially, my mum Sophie and the entire family for always being there throughout this journey.

ABSTRACT

In Western Kenya, malaria is a major cause of morbidity and mortality with more than 70% of the population at risk.Its decline over the last 25 years indicates a shift to older children majority of who are asymptomatic. Kenya uses artemisinin combination therapy (ACT) specifically artemether-lumefantrine (AL) as first line for treatment of malaria infections and sulfadoxine-pyrimethamine (SP) as prophylaxis in pregnant women. However, delayed parasite clearance has been observed in Kenya. Resistance to antimalarials specifically ACT's and SP is associated with various polymorphisms in *P. falciparum* multidrug resistance gene 1 (*Pf*mdr1), multidrug resistance-associated protein 1 (*Pf*mrp1), dihydrofolate-reductase (*Pf*dhfr), dihydropteroate-synthase (*Pf*dhps), and chloroquine resistance transporter (*Pf*crt) genes. While symptomatic malaria infections are recognized and treated, recent reports have revealed a largeproportion of asymptomaticinfections.A recent study in Kombewa indicated that some asymptomatic participants did not clear parasites or disrupt transmission in a large proportion of study population despite adequate treatment. Given the limited data on drug resistance in asymptomatic infections, molecular epidemiological studies of drug resistance are required to assess these infections. The first objective was to determine *Plasmodium* species prevalence (*P.falciparum, P.malarie, P.ovale wallikeri, P.ovale curtisi- Pf, Pm, Pow, Poc*), second objective was to determine frequency of anti-malarial resistance gene polymorphisms and third objective was toinvestigate gametocyte variability in symptomatic and asymptomatic infections. In a retrospective cross-sectional study molecular techniqueswere used toanalyze 230 archived whole blood samples collected between 2018 and 2021 in Kombewa under malaria epidemiology surveillance and malaria transmission study representing symptomatic and asymptomatic infection. The species composition(*Pf, Pm, Pow, Poc*) and gametocyte carriage (*Pfs16, Pfs25*) were determined using real-time polymerase chainreaction and analyzed using excel.Genotyping of *Pf*mdr1 86, 184 & 1246; *Pf*mrp1 437, 876 &1390; *Pf*dhfr 16, 22, 59 & 164; *Pf*dhps 436, 437 & 581, and *Pf*crt 72, 76, 271, 326, 356 single nucleotide polymorphisms (SNPs)were assayed using Mass ARRAY platform and analyzed against the reference 3D7 genome. Data and statistical analysis wasdone using excel and Chi square tests in STATA.Of the 230 samples analyzed, *Plasmodium* species prevalence was; *Pf* 64.35% (148/230), *Pm* 26.52%, (61/230), *Pow* 9.57% (22/230), *Poc* 6.09% (14/230).The symptomatic *Pf* comprised 70.59% (24/34), *Pm* 17.65% (6/34), *Poc* 11.76% (4/34), and *Pow* 8.82% (3/34) while for asymptomatic *Pf* 63.27% (63/196), *Pm* 28.06% (55/196), *Poc* 5.1% (10/196), and *Pow* 9.69% (19/196). Co-infections were higherfor *Pf*/*Pm*; symptomatic 11.76% (4/34), asymptomatic 19.39% (38/196) compared to all the other species combinations (≤6%).The *Pf*mdr1_184 harbored symptomatic 68.75% (11/16) and asymptomatic 52% (26/50) mutations, while *Pf*mdr1_1246 had 6% mutants in both symptomatic (1/16) and asymptomatic (2/30).For *Pf*mrp1 gene codon 437 had no mutations in symptomatic while asymptomatic had only one mutation (1/30). *Pf*mrp1 codon 876, symptomatic reported 47.05% (8/17) & asymptomatic 37.93% (11/29). *Pf*mrp1 1390 symptomatic had 6.67% (1/15) and asymptomatic 6.9% (2/29) mutations respectively. *Pf*dhfr codons 16 and 22 had no mutations for symptomatic and asymptomatic. *Pf*dhfr 59 & 164 had 88.24% (15/17) and 90.91% (30/33) mutants for symptomatic and asymptomatic respectively. For both symptomatic and asymptomatic *Pf*dhps codons 436, 437 and 581 did not reveal any mutants.The *Pf*crt gene, codons72, 76, & 356 did not have any mutations for either symptomatic or asymptomatic. *Pf*crt 326 & 371 had 3.23% (1/31) and 11.11% (4/36) mutations in asymptomatic only.Overall gametocyte carriage was 65.65% (151/230), symptomatic cases positives were*Pf*16 85.29 (29/34); *Pf*25 79.41% (27/34); *Pf*16*Pf*25 93.1% (29/29) while asymptomatic had *Pf*16 68.88% (135/196); *Pf*25 67.86% (133/196) &*Pf*16*Pf*25 86.1% (124/144). Even though proportional comparisons did not reveal statistical significance, this study was critical in revealing variations of *Plasmodium* species prevalence, frequency of drug resistance markers, and gametocyte variability in symptomatic and asymptomatic infections. Findings highlight the need for heightened molecular surveillance and management of malaria infectionsfor timely and informed interventions in all infections.

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LIST OF ABBREVIATIONS AND ACRONYMS

ACT	-	Artemisinin Combination Therapy
AL	-	Artemether Lumefantrine
ART	-	Artemisinin
AS	-	Artesunate
CQ	-	Chloroquine
DNA	-	Deoxyribonucleic acid
GMS	-	Greater Mekong sub-region
HDSS	-	Health Demographic Surveillance System
HF	-	Halofantrine
LUM	-	Lumefantrine
MALDI-TOF MS	-	Matrix-assisted laser desorption ionization time-of-flight mass spectrometry
MQ	-	Mefloquine
ML	-	Milliliters
µM	-	Micromolar
NaCl	-	Sodium Chloride
***Pf*crt**	-	*Plasmodium falciparum* chloroquine resistance transporter
***Pf*mdr1**	-	*Plasmodium falciparum* multidrug resistance
***Pf*mrp1**	-	*Plasmodium falciparum* multidrug resistance protein 1
***Pf*dhfr**	-	*Plasmodium falciparum* dihydrofolate reductase
***Pf*dhps**	-	*Plasmodium falciparum* dihydropteroate synthase
QN	-	Quinine
RDT	-	Rapid Diagnostic Test
RNA	-	Ribonucleic acid
RPM	-	Rotation Per Minute
RT-PCR	-	Real Time - Polymerase Chain Reaction
SNPs	-	Single Nucleotide Polymorphisms
SP	-	Sulphadoxine Pyrimethamine
STATA	-	Statistics and Data
WHO	-	World Health Organization

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CHAPTER ONE
INTRODUCTION

1.1 Background Information

Malaria is still considered to be a public health concern despite interventions with 249 million cases reported globally; the World Health Organization (WHO) African Region accounted for 94% of these including the 608,000 deaths(WHO, 2021a, 2023), with Kenya accounting for 2%. InKenyamore than 70% of the population isat constant risk of getting malaria as reported by the 2021 Kenya Malaria Indicator Survey.Kenya has several malaria epidemiological zones that includes; high lake endemic (including Kombewa), highland epidemic, moderate coast endemic, seasonal low transmission and low risk zones (Githinji *et al*., 2016) which presents as a challenge in malaria case management, interventions and control. Kombewa sub-county, located in western Kenya is classified as malaria holoendemic region with prerennial transmission. A study by Kapesa *et al.* showed that in 2016, malaria accounted for 29.9% of all outpatient visits, and 36.9% of hospital admissions with a 5.2% fatality ratein the sub-county. Asymptomatic infections were reported at >40% (Ondeto *et al*., 2022).This is a clear indication that malaria elimination strategies seem to have plateaued across sub-Saharan Africa despite sustained interventions (WHO, 2021a, 2023).Moreover,Lake Victoria region and the surrounding areas remain highly endemic with presence of symptomatic and asymptomatic infections but the true burden is underestimated because conventional diagnosis may miss a substantial amount of asymptomatic submicroscopicinfections(Idris *et al*., 2016; Imwong *et al*., 2014; Kapesa *et al*., 2018).Furthermore the emergence and transmission of drug resistant parasites, insecticide resistance and asymptomatic submicroscopic infections include some of the stumbling blocks thatchallenge the goal for malaria elimination globally and threaten the recent gains in malaria control(WHO, 2021a).

The contribution of asymptomaticparasite carriers in the persistent transmission of malaria is not well understood with few studies in Kenya comparing the symptomatic and asymptomatic infections.Studies have shown that asymptomatic infections are prevalent in endemic regions(Bousema *et al*., 2014; Dokunmu *et al*., 2019a; Idris *et al*., 2016; Lin *et al*., 2014; Lindblade *et al*., 2013) yet limited information is available about the *Plasmodium* species prevalence and parasite genotype circulating in this population. Challenges in detection and management of asymptomatic infections at the community level is attributed to the semi

convenient sampling of the symptomatic malaria(Kapesa *et al*., 2018)and diagnostic difficulties for the asymptomatic who mostly have sub-microscopic parasitemia and can be easily missed by microscopy which is the standard diagnostic method in most health care facilities(Andagalu *et al*., 2023; Andolina *et al*., 2021).This warrants the need for expansion of surveillance programs to include asymptomatic infections and incooperate use of molecular methods which are more sensitive for accurate assessment and evaluation of symptomatic and asymptomaticinfections as in the case of the current study.

Eight species of malaria parasite that are known to infect humans include the following; *P. falciparum, P. malariae*, *P. ovale curtisi, P. ovalewallikeri, P. vivax P. knowlesi*, *P. cynomolgi*, and *P. simium* with the last three being zoonotic (Tang *et al*., 2020). *P. ovale wallikeri, P. ovalecurtisi,* and *P. malariae* species consists of less than 10% of all infections and are found in many parts of sub Saharan Africa(WHO, 2016).Although they are not regarded as being virulent, infections with *P. ovale* and *P.malarie*can be chronic and are associated with kidney disease, anemia among other comorbidities (Hawadak *et al*., 2021; Hayashida *et al*., 2017). The *Plasmodium* species vary in virulence, with *P. falciparum* and *P. vivax* being the most virulent and prevalent globally (Visser *et al*., 2014). Although most studies focus on *P. falciparum* and *P.vivax* species, their have been reports of steady increase in malaria cases linked to *P.malarie* and *P. ovale* species in many parts of South America and sub-Saharan Africa in both symptomatic and asymptomatic infections(Hayashida *et al*., 2017; Woldearegai *et al*., 2019).However most programs focus on symptomatic leaving out asymptomatic infections and this raises concern for malaria control and elimination efforts. The sympatric co-existence of *Plasmodium* species may cause co-transmission of more than one species by the same mosquito during a blood meal or inoculation of various parasite species sequentially leading to superinfection (Akala *et al*., 2021; Tang *et al*., 2020). Moreover, the features associated with clinical malaria due to *P. ovale* species and *P.malarie* species are not well understood yet scanty data is available on the same (Hawadak *et al*., 2021). Previous study in Kombewa on therepeutic efficacy of ACT on non falciparum species revealed a prevalence of 28% of other *Plasmodium* species using molecular techniques that was missed by microscopy at enrollment among the symptomatic malaria (Chemwor *et al*., 2023). Futhermore another study in the same region on asymptomatic infections identified a large proportion of study participants who were continuously infected however they did not characterize the *Plasmodium* species prevalence

(Andagalu *et al*., 2023). This emphasizes the need for more studies using sensitive techniques on both symptomatic and asymptomatic infections to determine*Plasmodium*species prevalence as in these study for proper identification and tailored interventions inorder to achieve the malaria elimination goals.

The co-infections comprising of *P. falciparum*and *P. malariae* is significantly associated with reports of a decreased risk of presenting with fever at the clinic in a Kenyan study(Akala *et al*., 2021) thus remaining asymptomatic and undetected. Furthermore, *P.ovale* and *P.malarie* species are often misdiagnosed as *P.vivax* and *P.falciparum* respectively thereby complicating diagnosis and causing asymptomatic infectious reservoirs that may jeopardize malaria control and elimination efforts (Kotepui *et al*., 2020a, 2020b).Some studies indicate that *Plasmodium* species interactions modulate malaria transmissionfor specific species infections yet they remain undetected by conventional diagnostic methods mostly used in the healthcare facilities (Gnémé *et al*., 2013; Tang *et al*., 2020; Zimmerman *et al*., 2004). Recently recognized as an important malaria transmission reservoir, asymptomatic infections is a major hurdle for malaria elimination (Schneider *et al.,* 2007). Previous study in Kombewa region revealed increased prevalence of *Plasmodium* species however the study focused on symptomatic infections only (Akala *et al*., 2021). Control and elimination strategies of malaria therefore need to employ highly sensitive techniques to identify such reservoirs (Woldearegai *et al*., 2019).These findings underscores the need for attention towards all *Plasmodium* speciesin symptomatic and asymptomatic infection. Based on this,the current study determined the prevalence of *Plasmodium* species using sensitive RT-PCR for symptomatic & asymptomatic infections in Kombewa during 2018 – 2021 period.

Antimalarial drug resistance is attributed to single nucleotide polymorphisms (SNPs) in various *P.falciparum* genes, including: the multidrug resistant (*Pf*mdr1) gene on chromosome 5 and multidrug resistant protein 1 (*Pf*mrp1) (ATP-binding cassette transporters associated with artemisinin, amodiaquine, chloroquine, lumefantrine and mefloquine, resistance); the dihydrofolate reductase (*Pf*dhfr) gene on chromosome 4 (associated with pyrimethamine resistance); dihydropteroate synthetase (*Pf*dhps) gene on chromosome 8 (associated with sulphadoxine resistance) and chloroquine transporter resistance gene (*Pf*crt) on chromosome 7 (a drug-metabolite transporter associated with chloroquine resistance) (Njokah *et al*., 2016; Somé *et al.*, 2016).Antimalaria drug resistance has been observed to emanate in well-defined regions that

have distinct epidemiological zones (Takala-Harrison *et al*., 2015).The emergence of *P. falciparum* resistance to chloroquine (CQ) and later sulfadoxine-pyrimethamine (SP) was first reported in southeastern Asia and later spread to sub-Saharan Africa which harbors the greatest malaria burden (Maïga-Ascofaré & May, 2016). Following resistance to chloroquine and sulfadoxine-pyrimethamine, the Artemisinin Combination Therapies (ACTs) were introduced leading to a steep decline in malaria globally.Howeverreports of emergingantimalaria drug resistance and delayed parasite clearance remains one of the biggest challenges for malaria eradication programs worldwide(Imwong *et al*., 2017; Su *et al*., 2019). Decreased parasite susceptibily and resistance to artemisinin have been reported in Greater Mekong sub-Region (GMS) in Asia(Nsanzabana, 2019) and is associated with *Kelch 13* gene mutation in the propeller gene domain. In addition, studies have confirmed emerging *Kelch 13* gene mutation linked with artemisinin resistance in Rwanda (Achieng *et al*., 2020) and Uganda (Asua e*t al*., 2021; Balikagala *et al*., 2021)even though the exact resistance mechanism is not clearly understood.Although the reported *Kelch 13* gene mutation in Africa are associated with slow parasite clearance, ACTsare still in use and efficacious in Kenya, Uganda (Uwimana *et al*., 2020) and few other countries in the horn of Africa (WHO, 2020). Therefore, there is need for more studies and surveillance on the other antimalaria drug resistance genes (Nsanzabana *et al*., 2018). One of the most valuable methods used for assessing antimalarial drug efficacy is use of these molecular markers to detect and monitor drug resistant parasite (WHO, 2021a). Nevertheless, most of these studies focus on the profiles among the symptomatic malaria infectionsonly(Chebore *etal*., 2020; Eyase *et al*., 2013). Recent study on asymptomatic infections by Andagalu *et al* conducted in Kombewa ahigh malaria burden settings revealed that despite treatment with artemether-lumefantrine, theseasymptomatic infections were frequently positive for malaria raising the concern of poor response of asymptomatic infections to treatment (Andagalu *et al*., 2023). However the study by Andagalu did not conduct genetic characterization to determine the parasite drug resistance genotypes in these infections. Based on these findings, the current studydetermined the frequency ofmalaria drug resistance single nucleotide polymorphisms amongst symptomatic and asymptomatic malaria infections by genotyping of *Pf*mdr1, *Pf*mrp1, *Pf*dhfr and*Pf*crt genes using the iPLEX MassARRAY platform during the period of 2018 and 2021 in Kombewa sub county.These are validated markers associated with the current antimalarial used for treatment. This study was imperative to discern whether the

asymptomatic or symptomatic phase of the infections has a central reservoir of resistance role because monitoring the drug resistance to the available antimalarial drugs helps to implement effective drug policy (Antony & Parija, 2016).

Gametocyte carriage is important for assessment because it is the key stage that is transmitted by mosquitoes during its blood meal. Data from Senegal, Cameroon, The Gambia and Mali indicate that over 25% of individuals with sub-microscopic gametocytes were capable of infecting mosquitoes (Bousema *et al*., 2012). Studies on gametocyte carriage indicate that increasing proportions of gametocytes in malaria infections are submicroscopic and asymptomatic(Koepfli *et al*., 2017; Ouédraogo *et al*., 2018). However, a study in Kombewa Kenya by Andagalu et al indicated that individuals having submicroscopic parasitemia were at a lower risk of gametocytemia compared to those who had microscopic parasitemia yet they transmitted at a significantly higher rate. Moreover, no studies have compared the variability of gametocytes in symptomatic and asymptomatic malaria infections in this region therefore this study was paramount.These asymptomatic infections and gametocytes are usually challenging in their diagnosis as they occur at submicroscopic densities in older children and adults (Andagalu *et al*., 2023; Myers-Hansen *et al*., 2020; Walldorf *et al*., 2015) and can be easily missed by the conventional diagnostic tools in most health care facilities yet they act as infectious reservoirs for malaria transmission(Koepfli *et al*., 2021). Furthermore, most malaria surveys use light microscopy for investigating gametocyte carriage thus the true prevalence and contribution of sub-microscopic gametocyte carriage in symptomatic and asymptomatic infections maybe underestimated thereby having huge implications for the design and application of anti-malarial interventions (D'Alessandro, 2018; Galatas *et al*., 2016).

Achieving malaria elimination requires interrupting transmission as well as identifying and treating all carriers, including symptomatic and asymptomatic malaria infections that provide silent transmission reservoirs. Currently, gametocyte detection has been revolutionized by introduction of RNA-based molecular diagnostic assay (Gaur *et al*., 2017; Imwong *et al*., 2014; Tedla, 2019). Specific RNA transcripts including *Pfs25* expressed on female gametocytes, *Pfs16* (earliest marker of sexual stage developement) and *Pfs230* expressed on both sexes can be used for screening (Gaur *et al*., 2017; Gebru, Lalremruata, *et al*., 2017; Singh *et al*., 2020; Wang *et al*., 2020). Use of sensitive molecular techniques like PCR are critical in screening and diagnosis of these infections to give a true estimate and their overall contribution to the transmission of

malaria in symptomatic and asymptomatic infections. The current study used reverse transcriptasereal time quantitative PCR to investigate the variability of gametocytes by targeting *Pfs16* and *Pfs25* markers in symptomatic and asymptomatic infectionsin Kombewa within Kisumu County, Kenya during the period between 2018 – 2021.

Given that parasites with antimalarial drug-resistant genes have a higher probability of producing great numbers of gametocytes as compared to wild type parasites (Abdul-Ghani *et al*., 2015), transmission of drug-resistant genes through gametocytes is intensified hence the need for more studies on symptomatic and asymptomatic infections(Barnes *et al*., 2008; Méndez *et al*., 2002; Price *et al*., 1999).This data is essential for the evaluation and design of strategies to disrupt malaria transmission.

1.2 Statement of the Problem

Most programs and studies focus on *P.falciparum* and *P.vivax* globaly but *P.malarie* and *P.ovale* are not covered in many control programs. Recent studies have reported a steady rise in infections containing *P.ovale* species and*P.malarie*which are associated with chronic anaemia and other cormobidities yet limited data is available on these species. Microscopy and rapid diagnostic tests results in poor differenciation of the *Plasmodium* species for*P.ovale* and *P.malariae* leading to improper case management.Moreover gene polymorphisms associated with antimalaria drug resistance is reported to be on the rise hence the need for heightened surveillanceespecially with reports of resistance to ACTs in sEA and recently in Rwanda and Uganda.Furthermore, drug pressure is the primary factor responsible for the evolution and spread of drug-resistant parasites, and it is often more significant in symptomatic infections than in asymptomatic cases.However the asymptomatic reservoir plays a vital role in the transmission of drug-resistant parasites after clinical interventions, and thus, it contributes to the evolution and spread of antimalarial resistance as in previous cases for chloroquine and sulphadoxine-pyremethamine.In addition, large numbers of gametocytes are more likely to be produced in the presence of antimalarial drug-resistant parasites compared to wild type parasites therefore transmission of gametocytes harboring drug-resistant genes is enhanced yet microscopy may fail to detect submicrosopic gametocytes.Areas Surrounding Lake Victoria remain highly endemic, despite a declining transmission since 2000 (Collins *et al*., 2018; Kapesa *et al*., 2018, Idris *et al*., 2016 Takala *et al*., 2009) with studies reporting increased asymptomatic infections in endemic

areas(Niang *et al*., 2017). Use of molecular techniques inassessing the prevalence of *Plasmodium* species, frequency of antimalarial drug resistance markers and gametocyte variability in symptomatic and asymptomatic infections during the period of 2018 to 2021 was essentialas it is the first study to conduct side by side comparison in Kombewa region.

1.3 Objectives

1.3.1 Main Objective

To assess*Plasmodium* species prevalence, antimalarial drug resistance genes polymorphisms and gametocytesin symptomatic and asymptomatic infections in Kombewa, Kenya.

1.3.2 Specific Objectives

i. To determine prevalence of *Plasmodium* species in symptomatic and asymptomatic infections in Kombewa, Kenya.
ii. To determine the frequency of *Pf*mdr1, *Pf*mrp1, *Pf*dhfr, *Pf*dhps &*Pf*crt single nucleotide polymorphisms associated with antimalarial drug resistance in symptomatic versus asymptomatic infections.
iii. To determine the variability of gametocyte carriage in symptomatic and asymptomatic infections in populations in Kombewa within Kisumu County, Kenya.

1.4 Research Questions

i. What is the prevalence of *Plasmodium* speciesin symptomatic and asymptomatic malaria infections in Kombewa within Kisumu County, Kenya?
ii. What is the frequency of *Pf*mdr1, *Pf*mrp1, *Pf*dhfr, *Pf*dhps &*Pf*crt single nucleotide polymorphisms that are associated with antimalarial drug resistance in symptomatic and asymptomatic population in Kombewa Kisumu County, Kenya?
iii. What is the variability of gametocytes carriage in symptomatic and asymptomatic malaria infections in populations in Kombewa within Kisumu County, Kenya?

1.5 Research Hypothesis

i. Ho_1: There is no difference in the prevalence of *Plasmodium* speciesin symptomatic malaria and asymptomatic infections in Kombewa within Kisumu County, Kenya.

ii. Ho_2: The frequency of *Pf*mdr1, *Pf*mrp1, *Pf*dhfr, *Pf*dhps &*Pf*crt single nucleotide polymorphisms that are associated with antimalarial drug resistance are not different in symptomatic and asymptomatic infections in Kombewa Kisumu County, Kenya.

iii. Ho_3: There is no variability of gametocytes carriage in symptomatic malaria and asymptomatic infections in Kombewa within Kisumu County, Kenya.

1.6 Study Significance

The current study used real time-PCR to determine *Plasmodium* species prevalence in symptomatic and asymptomatic infections which is paramount for accurate diagnosis and subsequent case management of all malaria infections(Woldearegai *et al*., 2019). The WHO has put in place programs that focus on *P.falciparum* and *P.vivax* leading to a significant decline globaly but *P.malarie* and *P.ovale* are not covered in many control programs (Iwagami *et al*., 2017). Reports of rising prevalence of these non-falciparum species highlights the need for a focus on all *Plasmodium* species as opposed to targeted specific species inorder to determine their prevalence(Akala *etal*., 2021). Furthermore,recent study on asymptomatic infections reported increased frequency of malaria infections despite adequate treatment in Kombewa yet genotypes associated with drug resistance were not assessed (Andagalu *et al*., 2023). Therefore, the results from the current study are of great importance for determining the frequencies of the single nucleotide polymorphism in these genes following use of Mass ARRAY platform especially with recent reports of delayed parasite clearance and drug resistance to ACTs(Balikagala *et al*., 2021) in symptomatic and asymptomatic infections. In addition, a considerable proportion of malaria infections consist of gametocytes at various quantities however the conventional diagnostic tools may fail to detect submicroscopic gametocytes and asymptomatic infections yet a more robust sensitive tool applicable for clinical purposes is still lacking. Sensitive screening techniques i.e reverse transcriptase Real Time PCR as used in this study was crucial for accurate detection of submicroscopic parasitemia and asymptomatic infections subsequently resulting to improved estimation of the disease implication and better understanding as regards transmission dynamics of malaria in human populations. Asymptomatic infections have become increasingly prevalent in many endemic areas and is the parasite's best

asset for survival (Lin *et al*., 2014)yet they are not included in the malaria control and elimination programs. The assessment of symptomatic and asymptomatic infections using molecular techniques is critical for acquisition of precise data to guide the design and implementation of strategies for interrupting malaria transmission and for informing the outcome of malaria control measures. Moreover, strengthened focus on malaria eradication is needed for disruption of transmission by use of sensitive techniques for proper identification and treatment of both symptomatic and asymptomatic (Woldearegai *et al*., 2019)infections whereby molecular techniques used in this study were essential.

The findings from this study were essential in giving more insight on *Plasmodium* species prevalence, frequency of antimalaria drug resistant genotypes and the variability of gametocytes among symptomatic and asymptomatic infections as there are implications for understanding and forecasting their burden. This is key for enhancement and sustainability of the recent progress in malaria control and elimination whose efforts seem to have stalled.

CHAPTER TWO
LITERATURE REVIEW

2.1 Malaria Prevalence

In the previous decades malaria declined slightly from 238 million cases in 2000 to 229 million in 2019 globally with reports of regression and elimination in several regions including; Armenia, Argentina, Algeria, Morroco, Paraguay, Kyrgyztan and United Arab Emirates (WHO, 2015a). However, malaria is still considered to be a public health concern despite interventions whereby 249 million cases were reported globally and sub-Saharan Africa accounted for 94% of these including the 608, 000 deaths (WHO, 2021a, 2023).Slow progress in malaria decrease between 2019 and 2020 was reported in the World Health Organization (WHO) Africa region, where Kenya accounted for 2% of the burden (WHO, 2021a). One study in Kombewa region by Kapesa revealed that malaria accounted for 29.9% of all outpatient visits, 36.9% of hospital admissions with a 52% fatality rate in the Sub-County (Kapesa *et al*., 2018) predominantly in under five years old children. Despite declining malaria transmission, there are reports of resurgence by 56% in this region (Andagalu *et al*., 2023; Ondeto *et al.*, 2022).Malaria prevention efforts such as the deployment of the RTS, S/AS01, which is a pre-erythrocytic vaccine may not replace the use of antimalarial drugs. This is because studies have reported moderate vaccine efficacy particularly in malaria endemic regions (Takala & Plowe, 2009; WHO, 2016). Malaria continues to persist as a result of increasing cases of non-falciparum *Plasmodium* species(Akala *et al*., 2021), emerging cases of anti-malaria drug resistance and asymptomatic submicroscopic infections(Andagalu *et al*., 2022; Asua *et al*., 2021; Nguitragool *et al.*, 2017; WHO, 2017, 2018). Consequently,assessment of*Plasmodium* parasites in symptomatic and asymptomatic infectionsas in the case of the current study is important for precise case management and informed control strategies.

2.2 Symptomatic and Asymptomatic Infections

Symptomatic malaria is characterized by a high temperature of 38°C and above, muscle pains and generally feeling un-well, vomittingwith recurrent fever and chills, which often trigger treatment-seeking behavior once parasite replication becomes synchronous(WHO, 2015a). In some cases, the infection may result in parasitemia of different densities, without fever or other symptoms, in otherwise healthy individuals, therefore regarded asymptomatic or chronic infections(Ramaswamy *et al*., 2020). Asymptomatic infections have been reported in various

regions across the globe including; Solomon Island, Brazil, Peru and Colombia (Branch *et al*., 2005; Cucunubá *et al*., 2008; Laishram *et al*., 2012; Lee *et al*., 2010).In Africa, over 90% of asymptomatic infectionsare in older children and adults(Golassa *et al*., 2015a; Kapesa *et al*., 2018; Njama- Meya *et al*., 2004) including Western Kenya,with Kombewaregion having the highest prevalence of 40%(Ondeto *et al*., 2022) and also bearing the highest risk of asymptomatic infections(Baliraine *et al*., 2009; Kapesa *et al*., 2018; Ondeto *et al*., 2022). While a majority of studies and programs focus on symptomatic malaria which is well studied, the epidemiological variables that are linked to asymptomatic submicroscopic infections are not captured well in the malaria control programs (Golassa *et al*., 2015b; Lindblade *et al*., 2013). Furthermore, following recent reports of declines in clinical symptomatic malaria, studies indicate the increasing asymptomatic parasite carriers are key in maintaining transmission (Iwagami *et al*., 2017; Lover, Dantzer, *et al*., 2018; Niang *et al*., 2017; Sáenz *et al*., 2017). Sub-microscopic parasitemia are not easily detected by microscopy or RDT in asymptomatic individuals yet they serve as a silent infectious reservoir for transmission by anopheles mosquitoes and may advance to symptomatic malaria (Golassa *et al*., 2015b; Njama- Meya *et al*., 2004).Therefore sensitive diagnostic techniques are needed to determine their prevalence for infomed descion making and intervention strategies(Cheaveau *et al*., 2019).This study used sensitive molecular techniques to assess the *Plasmodium* species prevalence, frequency of drug resistance gene polymorphisim and gametocyte variability in symptomatic and asymptomatic infections in Kombewa a lake endemic malaria region inorder to inform malaria control strategies and timely intervention.

The WHO recommends diagnosis of malaria by microscopy (considered as the gold standard)and rapid diagnostic test (RDT) for patients with suspected malaria for confirmation before treatment(WHO, 2013). Microscopy requires high level of expertise inorder to achieve sensitivity that can be used for quantificationofthe parasite, species differentiation and parasite life cycle stage identification(Golassa *et al*., 2015a)however it can not detect low levels of submicroscopic asymptomatic parasitemia hence a substantial proportion is missed. The RDTs are much easier to use for detection ofspecific *Plasmodium* antigen from the parasite using one or more of the three antigen targets including lactate dehydrogenase (LDH),Histidine-rich protein 2(HRP2), and aldolase.The HRP2 is the most used target antigen broadly for malaria RDTs and is only expressed by *P. falciparum* while aldolase and LDH are expressed by all

Plasmodium species however they normally yield lower accuracy in diagnosis in the commercially available RDTs (Golassa *et al*., 2015a; WHO, 2013). Molecular techniques such as PCR are useful in the determination of the true burden of malaria among symptomatic and asymptomatic infections in endemic areas which is facilitated in this study for planning effective malaria control strategies.

Some studies have revealed that infections remain asymptomatic due to sustained submicroscopic peripheral parasitemia, (Kho *et al*., 2021; Pava *et al*., 2016) age and therefore immunity of the infected individual, and varying malaria pyrogenic threshold that is dependent on immunity. Moreover, untreated asymptomatic infections can end up into a chronic infection characterized by changes in the red cell precursors and high levels of erythrophagocytosis, anaemia and other associated commorbidities (Matangila *et al*., 2014). Malaria intervention and elimination efforts requires more than treatment of symptomatic clinical cases to focusing on community transmission by prompt identification and treatment of asymptomatic infections (Wu *et al*., 2015).This is key because the sensitivity of tests may be lower in the asymptomatic infections as compared to the symptomatic malaria. Elimination programs should therefore target both symptomatic and the challenging asymptomatic infectious reservoirs to rapidly eliminate the disease (Price *et al*., 2004). Consequently, this study used molecular techniques to determine prevalence of *Plasmodium* species, frequency of antimalarial drug resistance genes and gametocyte variability in symptomatic and asymptomatic infections in Kombewa Kisumu County, Kenya.

2.3 *Plasmodium* Species in Symptomatic and Asymptomatic Infections

2.3.1 *Plasmodium* Species Prevalence

Recent studies have revealed that different *Plasmodium* species composition also play a critical role in malaria infection (Akala *et al.*, 2021) yet limited information on their prevalence is available. In addition, studies have reported conflicting findings about *Plasmodium* species infection in asymptomatic infections and its role in symptomatic malaria, whereas some studies have found decreased *P. falciparum* parasitemia, (Bereczky *et al*., 2007; Males *et al*., 2008; Portugal *et al*., 2017; Sondén *et al*., 2015), some have indicated an increase in *P. falciparum* parasitemia in different populations (Eldh *et al*., 2020; Le Port *et al*., 2008; Liljander *et al*., 2011; Njama- Meya *et al*., 2004; Nsobya *et al*., 2004). Asymptomatic infection is prevalent in malaria

endemic areas and is mostly associated with high prevalence of anemia that varies from mild to moderate (Amato *et al*., 2017). Findings of infections comprising multiple *Plasmodium* species have been documented from various reports (Bruce *et al*., 2000; Ebrahimzadeh *et al*., 2007; Snounou, Pinheiro, *et al*., 1993). Moreover, studies have focused on *P.falciparum* and *P. vivax* because they are more virulent and prevalent however in recent years, their have been reports of increase in *P. ovale wallikeri*, *P. ovale curtisi* and *P.malarie* in many parts of South America and WHO African regionin symptomatic and asymptomatic infections yet they remain as neglected tropical diseases (Hayashida *et al*., 2017). In malaria endemic regions with consistently high transmission like Nigeria (Engelbrecht *et al*., 2000),Tanzania (Babiker *et al*., 1999) and partly Senegal (Babiker *et al*., 1999; Konaté *et al*., 1999), it has been estimated that between 70% and 90% of all infections harbors more than one *Plasmodium* species (Soulama *et al*., 2009). Another study in Lagos reported that of the seemingly healthy individuals, 20% (175/888) were infected with *Plasmodium* species (Phommasone *et al*., 2016). The most abundant and virulent malaria parasite in Kenya is *P. falciparum* which is associated with symptomatic malaria nevertheless, there have been reports of other *Plasmodium* species including *P. ovale wallikeri*, *P. ovale curtisi* and *P.malarie* with trends of increase in *P. ovale*(Akala *et al*., 2021; Chemwor *et al*., 2023)in Kombewa. However, scanty data is available on their prevalence in symptomatic and asymptomatic infections which is key for assessing malaria intervention programs and informing control strategies.

2.3.2 Clinical Symptoms

The clinical symptoms of malaria start between 7 to 85 days after initial inoculation depending on the infecting *Plasmodium* species nonetheless, parasitemia may also persist in non-immune individuals for as long as two, four or eight years before onset of symptoms depending on the *Plasmodium* species present (Greenwood *et al*., 2008). Some of the factors that influence progression and development of symptomatic malaria that are well studied include age, immunity and transmission intensity of the site (Buchwald *et al*., 2019; Worku *et al*., 2014).

The clinical characteristics associated with *P. ovale wallikeri*, *P. ovale curtisi* and *P.malarie* are poorly understood with very few reported studies available as compared to *P. falciparum* and *P.vivax*. Although they are not regarded as being virulent, *P. ovale* and *P.malarie* are associated with kidney disease and anemia among other comorbidities (Hawadak *et al*., 2021; Hayashida *etal*., 2017). Moreover, diagnostic challenges for species differenciation based on microscopy

whereby *P.ovale* and *P.malarie* species are often misdiagnosed as *P.vivax* and *P.falciparum* respectively thereby complicating diagnosis and causing asymptomatic infectious reservoirs that may jeopardize malaria control and elimination efforts (Kotepui *et al*., 2020a, 2020b). There have been reports that chemokines produced when the body mounts an immune response will interact depending on the *Plasmodium* species present whereby when an individual is reinfected by a second species, parasitemia due to the first species is down-regeulated (Bruce & Day, 2003). Some studies indicate that these interactions modulate malaria transmission (Gnémé *et al*., 2013; Tang *et al*., 2020; Zimmerman *et al*., 2004) for certain species in an infection and *Plasmodium* species co-infections may determine the resource allocation within a host depending on the species present yet scanty data is available on the prevalence of these species. This study provides valuable insight on the prevalence of *Plasmodium* species among symptomatic and asymptomatic infections during the period of 2018 to 2021 in Kombewa region.

2.3.3 Infection Diagnosis

These asymptomatic infections that comprise of different *Plasmodium* species, including those that can be easily missed by microscopy or Rapid Diagnostic Tests (Eichner *et al*., 2001; Tadesse *et al*., 2018) result in transmission to mosquitoes during blood meal (Wu *et al*., 2015). Moreover, even though RDTs takes a shorter time and can be easily used by nonskilled personell, they do not offer improved sensitivity as it decreases when parasitemia falls below 100 parasites per microlitre (Milne *et al*., 1994) in addition to false positivity due to antigens in circulation after the parasite has been cleared and failure to distinguish the different *Plasmodium* species present(Perandin *etal*., 2004). Several assays have been designed based on genus and species specific sequence of the single strand rRNA of the parasites 18S subunit rRNA(Balbir Singh *et al*., 1999; Kawamoto *et al*., 1996; Seesod *et al*., 1997).PCR assays are more sensitive over RDT and microscopy because they are highly specific and can detect as few as five parasites per microlitre(Moody, 2002) therefore they are idealfor identifying mixed species infections that can be overlooked by the conventional diagnosis used in most healthcare facilities(Balbir Singh *et al*., 1999; Snounou, Viriyakosol, *et al*., 1993).Most recent PCR based on fluorescent label tags enables continuous monitoring of PCR product formation as the assay is ongoing and has been adapted for detection of human *Plasmodium* parasite species that can be used for screening large number of samples(Calderaro *et al*., 2013; Lee *et al*., 2002).On this account, this study used real time PCR molecular assays as previously described by Calderaro and co-wokers to determine the

prevalence of *Plasmodium* species in symptomatic and asymptomatic infections in Kombewa a lake endemic malaria region in Kenya.

2.4 Malaria Drug Resistance Markers in Symptomatic and Asymptomatic Infections

2.4.1Resistance to Artemisinin Combination Therapy

The programs for malaria control recommends prophylaxis or therapeutic measures using chemotherapeutic agents (WHO, 2010, 2015b). However, implementation of these strategies are threatened by the inception and spread of drug-resistance (Happi *et al*., 2005; WHO, 2018). Presently, the global distribution and frequency of drug-resistant *P.falciparum* is variable and is partly a reflection of the transmission intensity and drug deployment patterns (Ippolito *et al*., 2021). Currently, in malaria endemic countries uncomplicated malaria is treated by use of artemisinin based combination therapies (ACTs) which is the first-line treatment (Ebenebe *et al*., 2018; Ishengoma *et al*., 2019; Sowunmi *et al*., 2016; WHO, 2018). Nevertheless, the parasites habouring *Kelch 13* gene mutations on chromosome 13 associated with resistance to artemisinin have been reported in Cambodia and subsequently in all countries of Greater Mekong Subregion (GMS) in Asia (WHO, 2010). Studies have confirmed emerging *Kelch 13* gene mutation linked with artemisinin resistance in Rwanda (Achieng *et al*., 2020) and Uganda (Asua *et al*., 2021; Balikagala *et al*., 2021). Moreover, although delayed clearance of the parasite following artemisinin treatment has been reported in several African countries including Kenya (Beshir et al., 2013) Nigeria (Sowunmi *et al*., 2016) and Angola (Plucinski *et al*., 2017), the association of *Kelch13* mutations with clinical resistance is not clear furthermore those mutations reported in South East Asia have not been observed in Africa commonly (Balikagala *et al*., 2017; Ménard *et al*., 2016; Muwanguzi *et al*., 2016). Such studies as that of Beshir highlighted above emphasize the need for surveys on other targets linked to antimalaria resistance. This will broaden strategies that prevent resistance and account for genomic evolution that greatly vary and differ as per geographic regions.

2.4.2 *Plasmodium falciparum* Single Nucleotide Polymorphisms

Antimalarial drug resistance is attributed to single nucleotide polymorphisms (SNPs) in various *P.falciparum* genes, including: the multidrug resistant (*Pf*mdr1) gene on chromosome 5 and multidrug resistant protein 1 (*Pf*mrp1) (ATP-binding cassette transporters associated with artemisinin, amodiaquine, chloroquine, lumefantrine and mefloquine, resistance); (Kavishe *et al*.,

2009; Sanchez *et al*., 2010) the dihydrofolate reductase (*Pf*dhfr) gene on chromosome 4 (associated with pyrimethamine resistance); dihydropteroate synthetase (*Pf*dhps) gene on chromosome 8 (associated with sulphadoxine resistance) (Gesase *et al*., 2009; Sridaran *et al*., 2010), and chloroquine transporter resistance gene (*Pf*crt) on chromosome 7 (a drug-metabolite transporter associated with chloroquine resistance) (Njokah *et al.*, 2016; Somé *et al*., 2016). Studies have reported resistance of the parasite to ACT patner drugs including aminoquinolines, amodiaquine and also chloroquine which is associated with polymorphisms in the *Pf*mdr1, *Pf*mrp1, *Pf*dhfr, *Pf*dhps and *Pf*crt genes (Sidhu *et al*., 2006; Venkatesan *et al*., 2014), however majority of these studies have been conducted on symptomatic malaria infections only leaving out asymptomatic infections. Moreover, reports of high frequency of positivity for malaria infection despite treatment in asymptomatic infections in Kombewa a malaria endemic region in Kenya is a cause for worry (Andagalu *et al*., 2023). It was therefore essential to evaluate the genotypes of parasite isolates circulating in symptomatic and asymptomatic infections because these parasites are often exposed to sub-optimal doses of anti-malarial drugs and the single nucleotide polymorphisms alter parasite susceptibility to ACTs (WHO, 2017).

Although previous studies have been conducted in Western Kenya on polymorphisms in these drug resistance markers, none has done a comparison of symptomatic and asymptomatic infections (Achieng *et al*., 2015; Chebore *et al*., 2020; Ngalah *et al*., 2015). Most studies focus on symptomatic malaria with scanty information on parasite genotypes in asymptomatic infections yet they arereported to be silent infectious reservoirs that enhance malaria transmission. Drug pressure is the primary factor responsible for the evolution and spread of drug-resistant parasites, and it is often more significant in symptomatic infections than in asymptomatic cases (Myers-Hansen *et al*., 2020) however, the asymptomatic reservoir plays a vital role in the transmission of drug-resistant parasites after clinical interventions, (Khalid, 2013)and thus, it contributes to the evolution and spread of antimalarial resistance as in previous cases for chloroquine (CQ) and sulphadoxine-pyremethamine (SP) (Hastings & Watkins, 2005; Myers-Hansen *et al.*, 2020). The emergence of *P. falciparum* resistance to chloroquine and later sulfadoxine-pyrimethamine was first reported in South East Asia and later spread to sub-Saharan Africa which harbors the greatest malaria burden (Maïga-Ascofaré & May, 2016). Continuous surveillance of single nucleotide polymorphisms associated with changes in antimalarial drug

sensitivity is essential for monitoring parasite genetic profile, conserving ACT efficacy and keeping track of selection(Venkatesan, 2014).

2.4.3 Artemether Lumefantrine and Sulphadoxine Pyremethamine Drug Resistance

In Kenya, artemether lumefantrine is used as first-line treatment for malaria and also sulphadoxine-pyrimethamine is administered for intermittent preventive treatment in pregnancy. Nonetheless, ACTs regimen of a short acting and a long acting drug comes with the potential of promoting resistance to the patner drugs which has a longer halflife and exists in the body system at subtherapeutic concentrations for several weeks (Venkatesan, 2014) and the persistence presence of SP resistance may render the drugs to be ineffective(Osborne *et al*., 2023).Findings show changing frequency of these genotypes in the population and persistence of others long after change of antimalaria drug policy(Eyase *et al*., 2013). In addition studies have revealed reports of synergism on the various drug resistance genotypes and continuous high prevalence of mutations in genes associated with antimalaria drug resistance(Bustamante *et al*., 2012; Gbotosho *et al*., 2012). However, most of these studies focus on the profiles among the symptomatic malaria infections only therefore this study was imperative to discern whether the asymptomatic or symptomatic phase of the infections has a central reservoir of resistance role.

The speed of mutants selections occurring within parasite populations is dependent on the pharmacokinetics of the antimalarial drug as well as the level of its utilization in thecommunity(Adamu *et al*., 2020).The development ofanti-malaria drug resistance is a big challenge in malaria endemic regions(Blasco *et al*., 2017; Cravo *et al*., 2015) and even though *in-vitro*and *in vivo*techniques can be used for assessing antimalria efficacy, it is expensive and skilled expertise is required. Consequently assessing antimalarial drug efficacy using these molecular marker analyses to detect and monitor drug resistant parasite provides a less costly alternative for assessing a large number of genes targets (Apinjoh *et al*., 2019)that providesequally valuable information for putting in place informed malaria control measures and strategies (WHO, 2021a).Rapid and timely molecular surveys such as the use of MassARRAY molecular techniques to establish the frequency of single nucleotide polymorphisms (SNPs)in antimalarial drug resistance genes as in this study is essential in order to inform regional drug policies and other stakeholders.

The MassARRAY platform utilizes the extension of PCR based single nucleotide base and has been previously described as being suitable for assaying SNPs linked to antimalaria drug resistance in Kenya (Yeda *et al*., 2016).The system uses a matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF) paired with single base extension PCR for single nucleotide polymorphism detection of the multiplex reaction (Syrmis *et al*., 2011) and is considered to be less expensive. The current study assessed the frequency of *Pf*mdr1 86, 184 & 1246; *Pf*mrp1 437, 876 &1390; *Pf*dhfr 16, 22, 59 & 164; *Pf*dhps 436, 437 & 581, and *Pf*crt 72, 76, 271, 326, 356 gene polymorphisms using MassARRAY platform in symptomatic and asymptomatic infections in Kombewa region during the period of 2018-2021 as tracking these molecular markers provides a tool for detecting emergence and distribution of parasites resistance genes (Nsanzabana *et al*., 2018).

2.4.5 *Pf*mdr1 Single Nucleotide Polymorphisms

The *Pf*mdr1 gene encodes for P-glycoprotein homologue 1 protein and is located on chromosome 5.It is a transmembrane protein with two domains that act as a site for ATP bindingin the digestive vacuole of the parasite (Antony & Parija, 2016). Moreover, antagonistic selective pressures following separate use of the antimalarials have been suggested by several genetic studies whereby the parasites harboring *Pf*mdr1 N86, 184F and D1246 genotypes are prevalent in African countries that use AL as the first line drug for treatment of malaria whereas those containing 86Y, Y184 and 1246Y alleles are prevalent in African countries that use artesunate-amodiaquine as first-line antimalarial drug (Okell, Reiter, Ebbe, Baraka, Bisanzio, Watson, Bennett, Verity, Gething, & Roper, 2018). Emergence of single nucleotide polymorphisms in *Pf*mdr1 N86Y, Y184F and D1246Y N86Y, Y184F and D1246Y is linked to the predominant use of artemether-lumefantrine and artesunate-amodiaquine in Africa for the treatment of uncomplicated malaria (Tukwasibwe *et al*., 2014). The changes in sensitivity to lumefantrine and amodiaquine which are partner drugs in artemisisnin based combination therapy is assessed by evaluating frequency of these mutations(Okell, Reiter, Ebbe, Baraka, Bisanzio, Watson, Bennett, Verity, Gething, & Roper, 2018). Findings from various studies have revealed that parasites carrying a combination of *Pf*mdr1 N86, 184F, and D1246 (NFD) portray reduced susceptibility to artemether-lumefantrine and that treatment with this drug can select for such haplotypes (Baliraine & Rosenthal, 2011; Sondo *et al*., 2016). In Uganda, Dokomajilar *etal*.,(Lee *et al*., 2006; Somé *et al*., 2010) highlighted that after treatment with artemether

lumefantrine, there was increased frequency of these *Pf*mdr1 alleles and the trend persisted in patients presenting with clinical failure. Moreover, in Tanzania, a high frequency of *Pf*mdr1 86Y, Y184 and 1246Y was observed by Humphreys *et al* in patients who failed treatment with amodiaquine but observed contradicting results in those who were treated with artemether-lumefantrine but failed treatment. Polymorphisms on *Pf*mdr1 in Nigeria is mostly found in Southern part and the frequency showed positive association between clinical failure and *Pf*mdr1 N86, F184 and D1246 alleles (Happi *et al*., 2009). The *Pf*mdr1 alleles carrying wild type N86 residue are associated with higher IC50 and IC90 values for mefloquine, lumefantrine and dihydroartemisinin while the alternative 86Y residue seems to confer increased resistance against amodiaquine and chloroquine(Veiga *et al*., 2016).

2.4.6 *Pf*mrp1 Single Nucleotide Polymorphisms

The *P.falciparum* multidrug resistance-associated protein 1 (*Pf*mrp1) is similar to *Pf*mdr1. MRP assists in the the transportation of organic anionic substrates and drug. The two mutations on positions Y191H and A437S in *Pf*MRP are associated with chloroquine and quinine resistance (Mu *et al*., 2003). It is involved in varying antimalaria response to drug in association with other transporters(Antony & Parija, 2016).

2.4.7 *Pf*dhps and *Pf*dhfr Single Nucleotide Polymorphisms

Resistance to sulphadoxine pyrimethamine occurs through mutations at the genes encoding *P. falciparum* dihydrofolate reductase (*Pf*dhfr) and dihydropteroate synthase (*Pf*dhps) (Gesase *etal*., 2009; Sridaran *et al*., 2010). Nonetheless sulphadoxine pyrimethamine treatment failure is strongly associated with single nucleotide polymorphisms commonly referred to as the triple mutations are found at *Pf*dhfr gene in three codons namely,N51I, C59R and S108N and double mutations in the *Pf*dhps at codons A437G and G540E and mutations at *Pf*dhps codon 613S have been reported and documented in Africa (McCollum *et al*., 2008) whereas the 436 and 581 mutations confer some degree of resistance. Recent findings have indicated that the presence of the 581G mutation may compromise continued IPTp using sulphadoxine pyrimethamine, however other studies suggest that IPTp with this drug seems to be effective even in regions with a high frequency of quintuple *P. falciparum* mutants (Spalding *et al*., 2010). This study analyzed *Pf*dhfr codons 16, 22, 59 & 164; *Pf*dhps codons 436, 437 & 581 to assess their frequency in the symptomatic and asymptomatic infections in Kombewa Kenya.The swift spread of *P. falciparum*

resistant genes that are associated with reduced susceptibility to sulphadoxine pyrimethamine in areas of malaria endemicity poses a major threat to the prevention of malaria in pregnancy where the WHO's 2021 world malaria report revealed that of the estimated 33.8 million in the WHO Africa region, one in three pregnancies (34 per cent, 11.6 million pregnancies) were exposed to malaria infection (Mockenhaupt *et al*., 2007; Staedke *et al*., 2001). Sulphadoxine pyrimethamine drug combination was adapted into the Kenyan National Policy in 1998 for use as Intermittent Preventative Treatment prophylaxis (IPTp) for malaria in pregnancy (Eijk *et al*., 2015) and also in Tanzania (Mikomangwa *et al*., 2020) however the continued selection pressure for mutations associated with sulphadoxine pyrimethamine resistance may render it to be ineffective.Previously, increased mortality in African children was attributed to *P. falciparum* resistance to chloroquine and later to sulfadoxine-pyrimethamine (Greenwood, 2004; Korenromp *et al*., 2003) therefore surveillance is warranted for detection of emerging resistance to enable timely intervention and averting similar occurence.

2.4.8 *Pf*crt Single Nucleotide Polymorphisms

Previously, chloroquine was the drug of choice for treating malaria until drug resistance emerged. The *Pf*crt gene on chromosome 7, is responsible for encoding a digestive vacuole transmembrane protein, and mutations on this gene are associated with chloroquine resistance (Bray *et al*., 2005). *P.falciparum* susceptibility to lumefantrine, mefloquine and other aryl amino alcohols, (Sisowath *et al*., 2005) are attributed to point mutations at *Pf*crt codon 76. There have been reports of re-emergence of chloroquine sensitive strains (Lu *et al*., 2017) however, it is uncertain if this re-emergence is a result the re-expansion of the susceptible parasites that survived the widespread drug pressure or reversal of mutations in formally resistant parasites in asymptomatic patients who serve as infectious reservoirs (Laufer *et al*., 2010). *Pf*crt mutations are associated with resistance to amodiaquine, chloroquine and lumefantrine;in Kenya the *Pf*crt gene particularly codons 72 – 76 CVIET and SVMNT haplotypes have been insinuated (Eyase *etal*., 2013) with the 76T point mutation being the main indicator for chloroquine resistance, while the SVMNT haplotype is associated with resistance to amodiaquine. Moreover, mutations on *Pf*crt gene impact the intracellular disposition of heme, which serves as the ART activator (Combrinck *et al*., 2013; Lewis *et al*., 2014; Ross *et al*., 2018)(Fidock *et al*., 2000). Parasites harboring CVIET are highly resistant to chloroquine but moderately resistant to amodiaquine (Eyase *et al*., 2013) whereas those parasites carrying SVMNT haplotype behave inversely and

are highly resistant to amodiaquine, but moderately resistant to chloroquine (Sa & Twu, 2010). Furthermore, *Pf*crt codon K76 is associated with emerging tolerance to lumefantrine (Lekana-Douki *et al*., 2011; Mwai *et al*., 2009; Sa & Twu, 2010).

2.4.9 Antimalaria Drug Resistance Genes and Synergism

Following the deployment of artemether lumefantrine and discontinuation of chloroquine in Western Kenya, there was drastic increases in the frequency of *Pf*mdr1 N86 and *Pf*crt K76 wild genotypes, however artemether lumefantrine was still efficacious despite the changes in this gene (Achieng *et al*., 2015). The EC50s for lumefantrine and mefloquine are reported to increase reciprocally with decrease in amodiaquine and chloroquine EC50s and these synergestic switching are linked to contrasting changes in the frequency of wild type versus mutant polymorphisms in *Pf*crt and *Pf*mdr1 genes (Eyase *et al*., 2013; Humphreys *et al*., 2007). Conformational changes in the transporter protein due to mutations on the *Pf*mdr1 gene causes decreased intracellular drug accumulation which play a critical role and effect on parasite resulting in variable parasite response to artemisinin, ACT and non-ACT (Dokunmu *et al*., 2019a; Gil & Krishna, 2017; Kaewpruk *et al*., 2016; Wurtz *et al*., 2014). There is known synergy between *Pf*crt and *Pf*mdr1 gene mutations (Dokunmu *et al*., 2019b) with reports of decreased in *vivo* and in *vitro* sensitivity to chloroquine in resistant isolates habouring the *Pf*crt 76T polymorphisim that is strongly associated with *Pf*mdr1 mutation in codon 86Y (Bustamante *etal*., 2012; Gbotosho *et al*., 2012).This highlights the need to investigate their frequencies for timely intervention to stop the expansion of resistant parasites habouring antimalarial gene mutations and for efficient malaria control strategies.

2.5 Gametocyte Variability in Symptomatic and Asymptomatic Infections

2.5.1 Gametocyte Prevalence

Malaria transmission in humans is dependent on the presence of mature gametocyte stages in the circulating peripheral blood and their release from asexual progenitors is variable among *Plasmodium* species. A significant portion of malaria infections habour gametocytes (Bousema *et al*., 2006; Koepfli *et al*., 2017). Studies across the globe have reported different gametocyte densities in Peru, Indonesia, and Papua New Guinea (Kosasih *et al*., 2021; Rovira-Vallbona *etal*., 2017). In sub-saharan Africa, a considerable proportion of the malaria infections contain gametocytes, Mali 89%, (Adomako-Ankomah *et al*., 2017), Burkina Faso 97% (Ouédraogo *et* al., 2008) and Malawi (Coalson *et al*., 2018). Reports from Senegal, Gambia, Mali and

Cameroon indicate that over 25% of individuals with sub-microscopic gametocytes are capable of infecting Anopheles mosquitoes (Bousema *et al*., 2012). In Western Kenya prevalence of gametocytes has been reported with varying densities in symptomatic and asymptomatic infections (Andagalu *et al*., 2023; Omondi *et al*., 2019; Touray *et al*., 2020). Gametocyte production is dependent on immunity among other factors whereby in symptomatic infections, the gametocyte infectivity might be reduced because of gametocyte-inactivating activity associated with inflammation and commitment to gametocytes might also be early or lower when parasites invest more in asexual multiplication compared with asymptomatic infections (Price *etal*., 1999; Shute & Maryon, 1951; Smalley *et al*., 1981). However limited studies have assessed the variability of gametocytes in symptomatic and asymptomatic infections yet they are key for transmission (Stone *et al*., 2015). Gametocyte screeningis key for malaria diagnosis, determining response to treatment and for characterizing the dynamics of malaria transmission (Babiker *et al*., 2008;WHO, 2018, 2021b). However, gametocytes normaly circulate at low density therefore detection using microscopy may capture only 50% of the carriers leaving out a considerable proportion of undetected gametocytes (Kepple *et al*., 2022; Wampfler *et al*., 2013). An infection with *P.falciparum* normally exhibits a gametocyte sex ratio of approximately one male to three or four females however the ratios varies by place, season and clone (Paul & Brey, 2003; Sowunmi *et al*., 2007; Talman *et al*., 2004). Moreover almost a quarter of the plasmodial genes are expressed during the sexual stages whereby specific RNA transcripts including *Pfs25* and *Pvs25* are expressed on female gametocytes while *Pfs230* are expressed on both sexes (Gebru, Ajua, *et al*., 2017; Khan *et al*., 2005). The *Pfs16* m RNA is the earliest sexual stage marker that is expressed in all gametocyte stages and *Pfs25* is is expressed in mature gametocytes(Babiker *et al*., 2008; Baker, 2010; Wang, 2020). Studies have reported that gametocytes can be intermittently seen and disappear therefore detection by microscopy is limited (Kepple *et al*., 2022; Okell *et al*., 2012). Furthermore, most malaria surveys use light microscopy for investigating gametocyte carriage thus the true prevalence and contribution of sub-microscopic gametocyte carriage in symptomatic and asymptomatic infections might be underestimated and this may have having huge implications for the design and application of anti-malarial interventions (D'Alessandro, 2018; Galatas *et al*., 2016). Use of sensitive molecular techniques like real time PCR are critical in screening and diagnosis of these submicroscopic parasitemia to give a true estimate and their overall contribution to the transmission of malaria (Omondi *et al*.,

2019), however limited studies have assessed the variability of gametocytes in symptomatic and asymptomatic infections. The current study investigated gametocyte variability in symptomatic and asymptomatic infections by targeting *Pfs16* and *Pfs25* markers using reverse transcription-quantitative PCR (RT-qPCR) in Kombewa Kisumu County, Kenya during the period of 2018 – 2021.

2.5.2 Gametocyte Density

The mature *Plasmodium falciparum* gametocytes appear and circulate for a few weeks 10–12 days following clearing of asexual parasites (Eichner *et al*., 2001). The gametocyte density is positively associated with parasite density within the asymptomatic population, and 83.8% of those who contribute to the infectious reservoir are individuals harboring microscopically detectable infections while the symptomatic malaria infections are uncommon comprising 0.6% only of the human infectious reservoir (0·040 episodes per person-year) (Koepfli *et al*., 2015). This very small contribution of symptomatic malaria infections to transmission is consistent with recent estimates for *P. falciparum* transmission in Ethiopia(Tadesse *et al*., 2018), but markedly different from findings in Thailand and Cambodia, where symptomatic malaria cases with high gametocyte densities were suggested to be more important than asymptomatic infections for maintaining malaria transmission (Lin *et al*., 2014; Vantaux *et al*., 2018). In Uganda, the episodes of symptomatic malaria are less likely to be gametocytaemic on presentation as compared with asymptomatic infections, suggesting that majority of symptomatic malaria cases present early, before the 9–12 days maturation of gametocytes is completed confirming that chronic asymptomatic infections with microscopically detectable parasitaemia are the most important drivers of transmission (Andolina *et al*., 2021; Rek *et al*., 2022). A recent study in Western Kenya by Andagalu et al indicated that individuals having submicroscopic parasitemia were at a lower risk of gametocytemia compared to those who had microscopic parasitemia (OR 0.04, $p < 0.001$) yet they transmitted at a significantly higher rate (OR 2.00, $p = 0.002$). Furthermore, given that parasites with antimalarial drug-resistant genes have a higher probability of producing great numbers of gametocytes as compared to wild type parasites (Abdul-Ghani *etal*., 2015), transmission of drug-resistant genes through gametocytes is intensified. Moreover, even though treatment is given, the impact of antimalarial drugs on gametocytes is dependent on the drug type and level of drug resistance (Beshir *et al*., 2013; Dunyo *et al*., 2006)hence the need to assess their variability in symptomatic and asymptomatic infections (Barnes *etal*., 2008;

Méndez *et al*., 2002; Price *et al*., 1999). However, limited studies have compared the variability of gametocytes in symptomatic and asymptomatic infections side by side in Kombewa and this formed the basis of the current study.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study Area

This study analyzed samples collected from Kisumu County specifically Kombewa in Western Kenya. Kombewa covers an area of about 369 km^2andis in a rural part on the north-eastern shores of Lake Victoria about 25 km from Kisumu town along Bondo-Kisumu Road (Figure 3.1). The area is positioned along Lake Victoria which is an important factor in etiology and transmission of malaria and is classified as lake endemic region(Peter Sifuna *et al*., 2014).

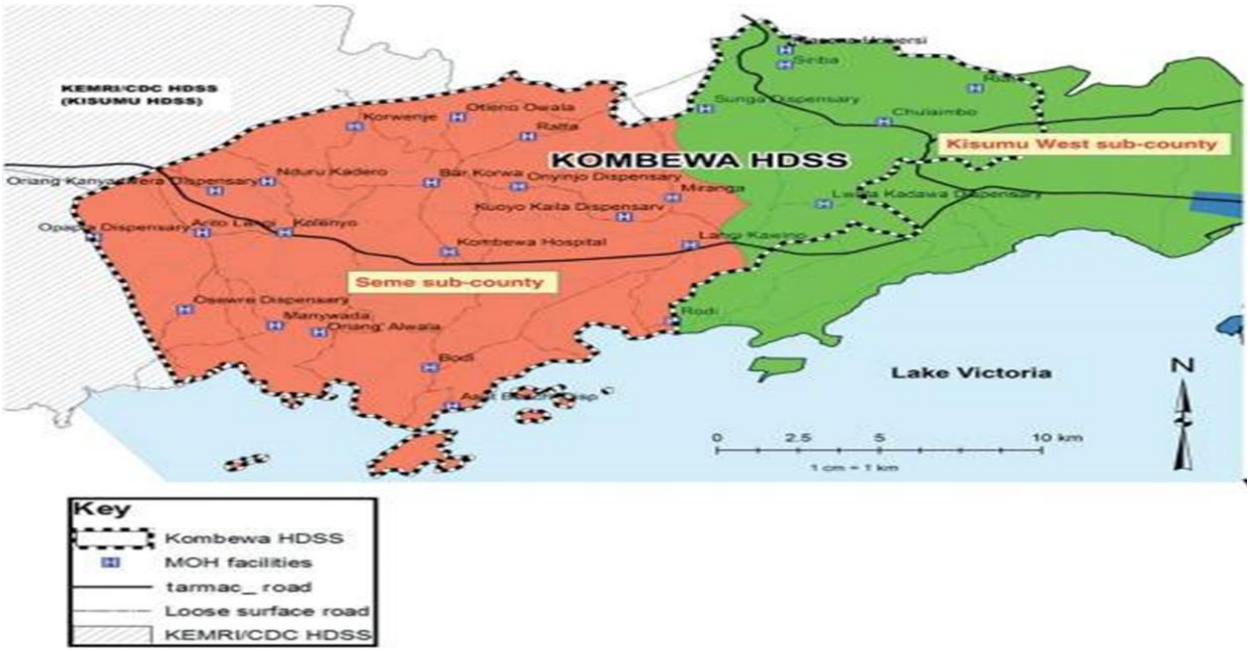

Figure 3.1: Map of Kombewa HDSS Area. Adapted from "Health & Demographic Surveillance System Profile: The Kombewa Health and Demographic Surveillance System (Kombewa HDSS)", by Sifuna *et al*., 2014.

3.2 Study Population

The study analyzed archived 230 samples that had been collected from symptomatic individuals seeking treatment at Kombewa hospital as symptomatic group(n=34) alongside samples obtained from healthy individuals from randomly selected households in Kombewa Community during random study visits**,** the asymptomatic group(n=196). The symptomatic samples were collected from Kombewa County hospital under ongoing epidemiology of malaria surveillance study while the asymptomatic samples were collected from community homesteads mapped in the Kombewa Health Demographic Surveillance System (HDSS)(P. Sifuna *et al*., 2014)under the

malaria transmission dynamics study from 2018 to 2021.Symptomatic infections were as per the classical clinical manifestation of malaria i.e a high temperature of 38°C and above, muscle pains and generally feeling un-well often characterized by recurrent fever and chills, which often trigger treatment-seeking behavior once parasite replication becomes synchronous. Asymptomatic infections presented as parasitemia of different densities, without fever or other symptoms, in otherwise healthy individuals. Therefore, asymptomatic infection referered to *Plasmodium* infections of any density in an individuals who did not have fever (< 37.5°C) or acute illness.

3.2.1 Inclusion Criteria

- **Symptomatic**

I. Samples collected from patients who presented with symptoms of malaria and had a positive parasitological test by microscopy or malaria Rapid Diagnostic Test.

- **Asymptomatic**

II. Samples collected from residents within the study area in good general health as evidenced by medical history and clinical examination by the clinician.

III. Samples positive for *Plasmodium* genus.

3.2.2 Exclusion Criteria

- **Symptomatic**

I. Samples with participant age below 10 years.

- **Asymptomatic**

II. Samples with incomplete demographic data.

1.1 Study design

The study design was a retrospective cross-sectional survey on archived samplesobtained from symptomatic and asymptomatic participants with infections between 2018 and 2021 and the assays were conducted at malaria drug resistance laboratory in Kenya Medical Research Institute,Centre for Global Health Research(KEMRI-CGHR) in Kisian.

3.3 Sample Size Calculation

Sample size for this study was calculated usingFisher's formula(Fisher, 1936)where malaria prevalence in Kombewawas estimated at18.4% and a population of 141,956(Peter Sifuna *et al*., 2014).

The following formula was used:

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

n = desired sample size (for population target >10,000)

Z = value for 95% confidence level (1.96)

p = approximate proportion of the population with the attribute in question (18.4%)

q =1-p

d^2 = standard error at 95% confidence limit (0.05)

$$\frac{1.96^2 x 0.184 x 0.816}{0.05^2} = 230$$

The proposed sample size was230.

The samples were randomly distributed between the symptomatic (n=34) and asymptomatic (n=196) infections.

3.4 Sample Accession, Retrieval and Processing

Permission was sought from the Principal Investigator of the parent study and the supervisor in line with the office protocol to access and retrieve the samples. Subsequently, whole blood samples that were collected in EDTA microtainers and stored at -80°C freezers were used for nucleic acid extraction. Consent for long term storage and characterization of samples in -line with emerging scientific innovations had been obtained from all the study participants prior to enrolment to the parent study. It is based on that consent that the samples remain available for additional studies. Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) was extracted from 200µl of whole blood samples that had been stored at -80^0C using Kingfisher™ Flex Purification System (Applied Biosystem) which is based on automated magnetic-particle processor. It was eluted at 100 µl and stored at -20^0C pending laboratory assays.

3.5 Laboratory Procedures

3.5.1 *Plasmodium* Genus Detection and Species Determination

Detection of *Plasmodium* using genus specific 18srRNA based Real-Time PCR assay was conducted using primers and other components of PCR in the assay as described by (Kamau *et al*., 2011). Amplification and real-time measurements were performed in the Applied Biosystems 7500 analytical PCR system with the following thermal profile for qPCR: 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 60°C. For qRT-PCR, a 30-min cycle at 50°C was added as

the initial step for the reverse transcription process. For the reaction, 1 µl of template was added to 9 µl of reaction master mix containing 1× QuantiTect Probe RT-PCR Master Mix (Qiagen), 0.4 µM each primer, 0.2 µM probe, and 4 mM MgCl2. For the qRT-PCR assay, 1 µl of QuantiTect RT Mix of enzymes was added to the reaction master mix. Subsequently, characterization of species composition assay for *Plasmodium falciparum* (*Pf*), *Plasmodium malariae* (*Pm*), *Plasmodium ovale curtisi* (*Poc*) and *Plasmodium ovale wallikeri* (*Pow*) using QuantStudio 6 Flex (Applied Biosystems) real-time PCR (qPCR) to confirm the species composition was done. All *Plasmodium* positive samples were assayed for species composition using a separate set of primers indicated in Table 3.1. The speciation assays for*Pf* and *Pm*was similar as the genus-specific Real-time PCR assay except for the primers used (similar PCR reaction components and conditions only). Genus-specific primers were replaced respectively by the species-specific primers. Specifically, FAL Reverse, FAL Forward, and FAL Probe were used for *Pf*identification while MAL Forward, MAL Reverse, and MAL Probe were used for *Pm*identification. All these primer sets were stored at -20^{0}C freezer and were removed only when the need to use arose. Detection of the two *P. ovale* sub species adopted previously described methods (Calderaro *et al*., 2012).

Table 3.1: **Primers and probes used for screening and diagnosis of *Plasmodium* species**

Primers Probes	Sequence 5'-3'	**Target Spp.**
PLU F	GCTCTTTCTTGATTTCTTGGATG	*Plasmodium* spp.
PLU R	AGCAGGTTAAGATCTCGTTCG	*Plasmodium* spp.
PLU P	ATGGCCGTTTTTAGTTCGTG	*Plasmodium* spp.
RNaSP F	TGTTTGCAGATTTGGACCTGC	Human RNase p
RNaSeP R	AATAGCCAAGGTGGAGCGGCT	Human RNase p
RNaseP P	TGCGCGGACTTGTGGA	Human RNase p
FAL F	ATTGCTTTTGAGAGGTTTTGTTACTT	*P. falciparum*
FAL R	GCTGAGTATTCAAACACAATGAACTCAA	*P. falciparum*
FAL P	CATAACAGACGGGTAGTCAT	*P. falciparum*
MAL F	GCATGGAATTTTGTTACTTTGA	*P. malariae*
MAL R	ATGCCTGTAGTATTCAACACAGAAAC	*P. malariae*
MAL P	TGTTCAAAGCAAACAGTTAAAACA	*P. malariae*
OVA F	TTTTGAAGAATACATTAGGATACAATTAATG	*P. ovale curtisi*
OVA R	CATGCTTCCTCTAAGAAGCTTTACAAT	*P. ovale*
OVA-V F	TTTTGAAGAATATATTAGGATACATTATAG	*P. ovale Wallikeri*
OVA-V R	CATCGTTCCTCTAAGAAGCTTTACAAT	*P. ovale Wallikeri*
OVA P	CCTTTTCCCTATTCTACTTAATTCGCAATTCATG	*P. ovale curtisi*
OVA-V P	CCTTTTCCCTACTTAATTCGCTATTCATTG	*P. ovale Wallikeri*

3.5.2 Gametocyte Detection

Diagnosis of gametocytes specifically*Pfs16* (early stage) and*Pfs25* (late stage) detection was carried out following RNA extraction using Applied Biosystems QuantStudio 6 Flex Real-Time PCR System.Specific primers and probesfor *Pfs16* and *Pfs25* wereusedto diagnose for the presence of gametocytes (Table.3.4).This was done using a one-step RT-PCR that enabled amplification of template RNA for the detection of gametocyte. Briefly, the assay consisted of a duplex assay encompassing 0.6 µl of AgPath-ID™ One-Step RT-PCR Reagent (Applied Biosystems, Foster City, California USA), 2 µl of the RNA template, 7.5 µl 2X RT-PCR Buffer, 0.4 µl of each primer and probe and 0.5 µl of nuclease-free water. The PCR cycling conditions included an initial cDNA synthesis step at 50°C for 10mins, followed by a PCR initiation step at 95°C for 5 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds then annealing and elongation steps for 30 seconds at 60°C. A positive gametocyte sample was determined to be either having amplified either one or both of the two targets, i.e. *Pfs16* and *Pfs25*.

Table 3.2: **Primers and probes used for gametocyte detection and amplification**.

Pf16 F	ATGCTTATATTCTTCGCTTTTGCA
Pf16 R	AATTCTAATACGACTCACTATAGGGAGAAGGGCGGGCTTTTTTGCTTTGT
Pf16 P	6FAM-AACCTG GTATTATCAGATGCAAATG-MGB
Pf25 F	CAGATGAGTGGTCATTTGGAATG
Pf25 R	AATTCTAATACGACTCACTATAGGGAGAAGGCTCCACATGGTTTATTTAC AGTCTTTTC
Pf25 P	VIC-CCCGTTTCATACGCTTGTAA-MGB

3.5.3 Genotyping for Malaria Drug Resistance Markers using Mass ARRAY

The alleles were determined by PCR-based single-base extension on Sequenom Mass ARRAY platform which uses matrix-assisted laser desorption ionization time-of flight mass spectrometry (MALDI-TOF MS) (Agena Biosciences, San Diego, CA, USA) . Extension of single bases based on PCR on Sequenom Mass ARRAY platform were used for analyzing the *Pf*mdr1, *Pf*mrp1, *Pf*dhps, *Pf*dhfr and *Pf*crt genes .Mass ARRAY assay for SNP genotyping using genomic DNA was done to amplify selected loci and polymorphisms in the*Pf*mdr1 86, 184 & 1246; *Pf*mrp1 437, 876 &1390; *Pf*dhfr 16, 22, 59 & 164; *Pf*dhps 436, 437 & 581, and *Pf*crt 72, 76, 271, 326, 356 single nucleotide polymorphisms (SNPs) as shown below.

3.5.4 First PCR Amplification

This PCR was for primary amplifications of the targeted gene of interest using specific primers. Briefly, 0.5µM each primer mix was prepared, comprisingforward and reverse primers for each gene target in an Eppendorf tube including the dNTPs, $MgCl_2$, enzyme and nuclease free water to make up the master mix and then mixed well by vortexing. Subsequently, 4 µl of the master mix prepared wastransferred into each well of the 0.2 conventional PCR plate followed by 2 µl of the DNA template. An adhesive sealer was used to cover the plates followed by centrifugation (Eppendorf). Amplification process was done using GeneAmp 9700 thermocycler (Applied Biosystems) using thermocycling conditions as follows: 95ºC for 2 minutes followed by 44 cycles of 95ºC for 30 seconds, 56 ºC for 30 seconds, 72ºC for 60 seconds, 72ºC for 5minutes and the final hold at 10ºC.

3.5.5 Shrimp Alkaline Phosphatase (SAP) Clean up PCR

After primary PCR, 2 µl of the ready-madeSAP master mixwas dispensed into the plate having samples, sealed, and centrifuged. This assay was used for removal of unwanted or unused reagents e.g.,dNTPs and it was done by incubating the plate in GeneAmp 9700 thermocycler (Applied Biosystems) at 37ºC for 40 minutes followed by 85ºC for 5 min and a 10ºC hold.

The SAP enzyme was used to dephosphorylate unincorporated nucleotides to prevent further reaction or addition/ elongation of chain.

3.5.6 Second iPLEX PCR

Multiplexed primer extension reaction was performed using mass-modified nucleotides. The iPLEX master mix was prepared by adding an adjusted multiplex of the target gene sequence specific primers, mass-modified nucleotides, iPLEX buffers, enzymes, and the terminator. 2 µl of this master mixwasadded into the plate having the samples mixed, sealed, vortexed and the plate centrifuged. The reaction occurred following incubation of the plate in GeneAmp 9700 thermocycler (Applied Biosystems) using the following conditions: 94ºC for 30 seconds, 45 cycles at 94ºC for 5 seconds, 52ºC for 5 seconds 80ºC for 5 secondsand 10ºC to infinity. This PCR was important for Addition Of The Mass-Modified Nucleotides.

3.5.7 Resin Clean up Conditioning

Clean up conditioning was performed by spreading clean resin paste into a 96-well dimple plate followed by incubation period for 10minutes at room temperature to dry. Subsequently, 42 µl of

nuclease free water was added into each well of the sample plate. Finally, the dimple plate with the dried resin was inverted into the sample plate and gently tapped on topfor the resin to drop into the wells containing the samples. The plate content was then sealed and mixed by rotating for 30 minutes followed by spinning down at 3000rpm for 5 minutes. This resin treatment was done to remove excess salts.

3.5.8 Sample Spotting on Mass ARRAY Chip and Loading onto Mass ARRAY Analyzer

The reaction product was dispensed onto a Spectro chip using a Nano dispenser. The chip was loaded and run on a Mass ARRAY Typer workstation (Agena Bioscience, Inc.) where detection of extension products occurred using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Agena Biosciences, San Diego, CA, USA). Analysis of genotyping results was carried out using Spectro Typer 4.0 software automated allele calling. Summary statistics was performed in excel file.

3.6 Data Management

The speciation and gametocytes qualitative PCR data was entered into excel spread sheet and percentage frequency was calculated to determine the species composition and gametocyte carriage for the two groups. Tables and Graphs (by MS Excel) were generated to show the percentage prevalence and frequencies of various variables. The data were exported to STATA version 12.0 for analysis of the variables: 1) Specific objective 1: the Chi-square test was used to test if there is a difference in *Plasmodium*species prevalence between symptomatic and asymptomatic infections; 2) Specific objective 2& 3: MassArray typer version.4.0 was used in SNP genotype calling against the reference 3D7 genome. The proportions of variables and parameters comparisons were assessed by Chi square tests using STATA version 12.0 forSingle Nucletide Polymorphisms (SNPs) and gametocyte carriage in the symptomatic and asymptomatic infections. All the statistical analyses were performed ata 5% significance levelwiththe Confidence Interval (CI) set at 95%; the statistical significance was defined as $p<0.05$. The data is presented in tables and graphs.

3.7 Ethical Consideration

All the sample IDs were assigned unique identifiers for data protection and confidentiality. Moreover, the sample IDs are not linked to any personal identifiable information of the participant. Permission to carry out this study wassought from Maseno University Scientific and

Ethics Review Committee (MUSERC) approval number MUSERC 01260/23. Ethical approval was acquiredfrom theHuman Subjects Protection Branch, of the Walter Reed Army Institute of Research (WRAIR #2454) and the Scientific and Ethics Review Units (SERU) at the Kenya Medical Research Institute (SERU #3628)for symptomaticand WRAIR #2739 / KEMRI #4082for the asymptomatic studies (Appendix 1).NACOSTI licence and approval was obtained to conduct the study (Appendix 2). Informed written consent was obtained from the participants and parents or legal guardians of the children who were enrolled in the study (Appendix3). The risks and how they were minimized as well as the benefits of the study and how the obtained data was stored were described in the consent forms (Appendix3). Informed assent was also obtained (Appendix 4).

CHAPTER FOUR

RESULTS

4.1 Participants Demographics

A total of 230 samples (34 symptomatic &196 asymptomatic)enrolled in malaria surveillance and transmission dynamics studies from the year 2018 – 2021 were analyzed.The females were marginaly less than the males (Table 4.1). A majority of the population was adults and the mean temperature was slightly variable;fever and chills characterized the symptomatic infetions(Table 4.1).

Table 4.1: Participants demographics

General characteristics	Symptomatic(n=34)	Asymptomatic(n=196)
Gender		
Female	16 (47%)	91 (46%)
Male	18 (53%)	105 (54%)
Mean age years (SD, Range)	17.1 (7.2, 10-48)	33.9 (8.8, 18-55)
Mean weight Kgs (SD, Range)	51.5 (14.6, 20-75)	63 (9.6, 38-100)
Mean temperature (^{o}C) (SD, Range)	37.6 (0.9, 36-40)	36.4 (0.3, 35-37)

The demographic data is presented as percentages and means including standard deviation (SD) and the minimum and maximum range.

4.2 *Plasmodium* Species Prevalence in Symptomatic and Asymptomatic Infections

The overallspecies prevalence was 73.91% (170/230)whereby four species were detected as follows; *Pf*64.35% (148/230), *Pm*26.52% (61/230), *Pow*9.57% (22/230), *Poc*6.09% (14/230).Mixed species coinfections werehigher for *PfPm* 18.26% (42/230) followed by *PfPow* 3.91% (9/230) and *PfPoc*2.61% (6/230), the remaining compositions were <1%.Furthermore, thespecies prevalence was variable in both symptomatic and asymptomatic infections.For the symptomatic,*Pf* comprised 70.59% (24/34), *Pm* 17.65% (6/34), *Poc* 11.76% (4/34), and *Pow* 8.82% (3/34) while for asymptomatic *Pf* 63.27% (63/196), *Pm* 28.06% (55/196), *Poc* 5.1% (10/196), and *Pow*9.69%(19/196) however the p-values were not significant(Table 4.2).Co-infections were higherfor *PfPm*;symptomatic11.76% (4/34), asymptomatic 19.39% (38/196) compared to all the other species combinations (≤6%)(Table 4.2).

Table 4.2:*Plasmodium* species prevalence in symptomatic and asymptomatic infections

***Plasmodium* species**	**Symptomatic (n)%**	**Asymptomatic (n)%**	**p-value**
Plasmodium falciparum	(24/34) 70.59%	(63/196) 63.27%	0.411
Plasmodium malariae	(6/34) 17.65%	(55/196) 28.06%	0.204
Plasmodium ovale curtisi	(4/34) 11.76%	(10/196) 5.1%	0.134
Plasmodium ovale wallikeri	(3/34) 8.82%	(19/196) 9.69%	0.904
PfPm	(4/34) 11.76%	(38/196) 19.39%	0.288
PfPoc	(2/34) 5.88%	(4/196) 2.04%	0.195
PfPow	(2/34) 5.88%	(7/196) 3.57%	0.521
PmPoc	(0/34) 0	(1/196) 0.51%	0.676
Pmpow	(1/34) 2.94%	(0/196) 0	0.016
PfPmPoc	(1/34) 2.94%	(2/196) 1.0%	0.362
PfPmPow	(0/34) 0%	(4/196) 2.04%	0.401

Comparison of single and multiple *Plamodium* species prevalence in symptomatic and asymptomatic infections. Chi square test was used for statistics.

4.3 Frequency of *Pf*mdr1, *Pf*mrp1, *Pf*dhfr, *Pf*dhps & *Pf*crt Single Nucleotide Polymorphisms in Symptomatic and Asymptomatic Infections

A subset of of 40 samples that were positive for *Plasmodium falciparum*speciesby real time PCR with cycle threshold of 30 and below was analyzed for the antimalarial drug resistance genotypes.The successful assays havinggood qualitycalls were included in the final analysis (Figure 4.1). Mixed/heterozygous genotypiccalls were considered as mutations.

4.3.1 *Pf*mdr1 Codons 86, 184 & 1246 SNPs

Successful sequences for *Pf*mdr1_86 did not have any mutations in symptomatic and only 5% (1/17) mutation was seen in asymptomatic. *Pf*mdr1_184 harbored symptomatic 68.75% (11/16) and asymptomatic 52% (26/50)mutations, while *Pf*mdr1_1246 had 6% mutants in both symptomatic (1/16) and asymptomatic (2/30).

4.3.2 *Pf*mrp1 Codons 437, 876 and 1390 SNPs

For this gene*Pf*mrp1 437 had no mutations in symptomatic while asymptomatic had only one mutation 3.23% (1/30). *Pf*mrp1 codon 876,symptomatic reported 47.05% (8/17)and asymptomatic 37.93% (11/29). *Pf*mrp1 1390 symptomatic had 6.67% (1/15) and asymptomatic 6.9% (2/29) mutations respectively.

4.3.3 *Pf*dhfr Codons 16, 22, 59, 164 and *Pf*dhps Codons 436, 437, 581 SNPs

*Pf*dhfr codons 16 and 22 had no mutations for symptomatic and asymptomatic.*Pf*dhfr codon 59 revealed88.24% (15/17) and 90.91% (30/33) mutantswhile*Pf*dhfr codon164 revealed6.67% (1/15)and 3.13% (2/32) mutants in symptomatic and asymptomatic, respectively.

For both symptomatic and asymptomatic,*Pf*dhps codons 436, 437 and 581 did not reveal any mutants.

4.3.4 *Pf*crt Codons 76, 271, 326, 356, 371

*Pf*crt codons76, & 356 did not have any mutations for either symptomatic or asymptomatic. *Pf*crt 326 & 371 had 3.23% (1/31) and 11.11% (4/36) mutations in asymptomatic only.

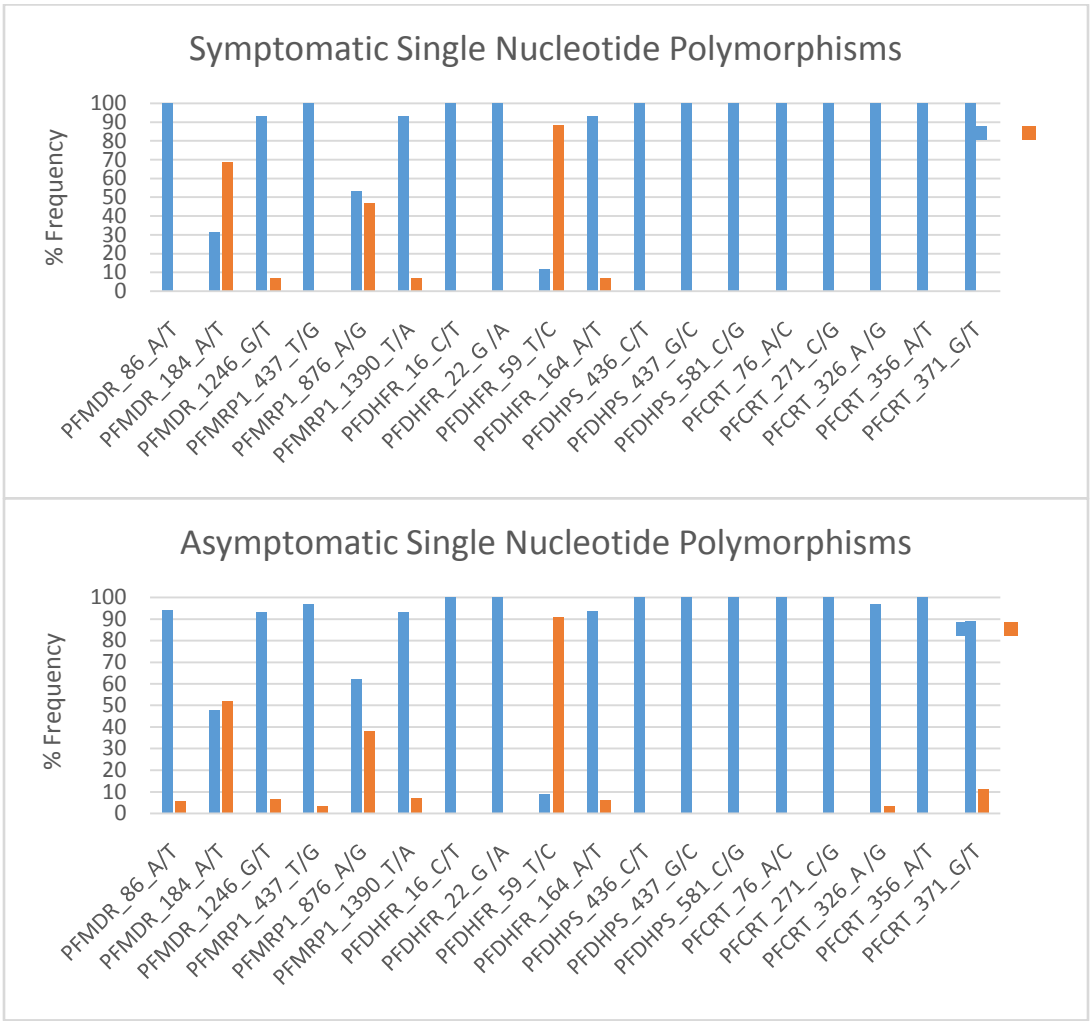

Figure 4.1: **Frequency of polymorphisms in *Pf*mdr1, *Pf*mrp1, *Pf*dhfr, *Pf*dhps &*Pf*crt genes associated with antimarial drug resistance among symptomatic and asymptomatic infections.**

4.4 Variability of Gametocytes in Symptomatic and Asymptomatic Infections

Overall gametocyte positives detected was 65.6% (151/230). The symptomatic cases revealed positives for *Pfs16* 85.29% (29/34); *Pfs*25 79.41% (27/34); *Pfs*16*Pfs*25 79.41% (27/34) while asymptomatic had *Pfs*16 68.88% (135/196); *Pfs*25 67.86% (133/196) &*Pfs*16*Pf*25 63.26% (124/196)even though it was not statistically significant (Table 4.3).

Table 4.3: **Gametocyte carriage in symptomatic and asymptomatic infections**

Gametocytes	Symptomatic (n)%	Asymptomatic (n)%	p-value
*Pfs*16	(29/34) 85.29	(135/196) 68.88	0.051
*Pfs*25	(27/34) 79.41	(133/196) 67.86	0.176
*Pfs*16*Pfs*25	(27/34) 79.41	(124/196) 63.26	0.302

Prevalence of gametocyte carriage in symptomatic and asymptomatic infections. Comparison of the proportions was done using Chi-square test in STATA.

CHAPTER FIVE

DISCUSSION

5.1 *Plasmodium* Species Prevalence in Symptomatic and Asymptomatic Infections

This study aimed to assess the prevalence of *Plasmodium* species, frequency of antimalarial drug resistance markers andtheir role in transmission among the symptomatic and asymptomatic infections. The global burden of malaria has reduced significantly due to the efforts made in malaria control(WHO, 2021b) which focuses mostly on *P.falciparum and P.vivax* however, there have been reports of rising prevalence of non-falciparum species.All samples analyzed in the current study were positive for *Plasmodium* genus and PCR assays revealed presence of four species in both symptomatic and asymptomatic infections. The most prevalent species was *P.falciparum*at 64.35%and this is in line with other studies followed by *P. malarie, P.ovale wallikeri* and the least was*P.ovale curtisi*(Dao *et al*., 2023). The prevalence of *Plasmodium* species co-infections was highest for *PfPm*at 18.26% followed by *PfPow*and *PfPoc*. Previous studies have reported that*P.falciparum* co-infection with *P. malariae*is common and is significantly associated with a decreased risk of presenting with fever at the clinic in Kenya (Akala *et al*., 2021)similar to a study in Mali (Dao *et al*., 2023)thus remaining asymptomatic and undetected. This auger well with our study whereby co-infections comprising of *PfPm* prevalence was higher for asymptomatic19.39% than the symptomatic 11.76% malaria infectionsalthough it was not statistically significant.

The marginally higher prevalence of *P. malariae* in asymptomatic than symptomatic in the current study may suggest that although former population (asymptomatic) may not present at the hospital, they may be affected by the comorbidities associated with this speciesincluding anaemia and kidney disease (Langford *et al*., 2015). The ability to sustain prolonged and low level parasitemia that is linked to increased burden of anaemia (Woldearegai *et al*., 2019)and subsequent hospitalization can be ascribed to *Plasmodium malariae* species which accounts for approximately 10% of clinical malaria,(Douglas *et al*., 2013; Langford *et al*., 2015), including tropical splenomegaly(Kotepui *et al*., 2020a; Leoni *et al*., 2015). The two sub species of *P. ovale*are characterized by varying epidemiology(Sutherland, 2016)whichare often misdiagnosed by microscopy in clinics and hospitals as other species, suggesting underreporting(Hawadak *et al*., 2021; Kotepui *et al*., 2020b).This study revealed the presence of both *P. ovale* species with *P. ovale walllikeri* being slightly higher in asymptomatic than symptomatic population.There

have been reports of increasing *P.ovale* species over time in both symptomatic and asymptomatic infections(Akala *et al*., 2021; Hawadak *et al*., 2021)and this can be attributed to the increased use of more sensitive techniques(WHO, 2017).

Following a four year study conducted in Burkina-Faso between 2007 and 2010, it was revealed that *P.ovale* species increased fourfold while *P.malarie* increased fifteenfold (Gnémé *et al*., 2013), similarly Democratic Republic of Congo reported increase in *P.ovale* species (Mitchell *etal*., 2021) after use of PCR which is a more sensitive method. A study done in Myanmar on asymptomatic infections(Nyunt, Shein, *et al*., 2017) revealed mixed *Plasmodium* species similar to the current study with microscopy and Rapid Diagnostic Test missingmajority of these asymptomatic infections that were eventually detected by PCR thus emphasizing the need for sensitive molecular techniques for screening and diagnosis of these sub-microscopic malaria infections.Furthermore, the Myanmar study reported that these asymptomatic infections were massively distributed in the study region a majority being males who were most affected (19/28, 67.8%).Similarly , another study in Kombewa Kenya on mixed *Plasmodium* species among symptomatic participantsrevealed co-infections that contained *P. falciparum* and other species which were not detected by microscopy on initial day of screening (Chemwor *et al*., 2023).Data on the contribution of asymptomatic infections to malaria transmission is limited(Lin *et al*., 2014) and the presence of varying *Plasmodium*species reported in the current study for both symptomatic and asymptomatic may indicate that they play an important role in maintaining malaria transmission in this holoendemic region.

There have been reports that chemokines produced when the body mounts an immune response will interact depending on the *Plasmodium* species present and some studies indicate that these interactions modulate malaria transmission (Gnémé *et al*., 2013; Tang *et al*., 2020) for certain species in an infection. Furthermore, *Plasmodium* species co-infections may determine the resource allocation within a host depending on the species present (Mideo & Day, 2008; Reece *etal*., 2010), *P. falciparum* is a generalist consuming all types of red blood cells whilst *P. ovale* and *P. malariae* are specialists with the latter being a heavy consumer of mature red blood cells while the former requires young red blood cells. Different specialized forms are achieved during parasite replication inside the host and transmission between hosts and resource allocation is also variable (Koella & Antia, 1995; Mideo & Day, 2008; Reece *et al*., 2010). Based on this results,

determination of species prevalence in symptomatic and asymptomatic infections is essential for informing malaria control strategies and timely case management.

5.2 Frequency of *Pf*mdr1, *Pf*mrp1, *Pf*dhfr, *Pf*dhps & *Pf*crt Single Nucleotide Polymorphisms in Symptomatic and Symptomatic Infections

In this study, sequences for *Pf*mdr1 N86 did not have any mutations in symptomatic and only (1/17) mutation was seen in asymptomatic infection. The high prevalence of the wild type N86 allele in this study is similar to a Nigerian study (Idowu *et al*., 2019) and may imply decreased susceptibility to artemeter lumefantrine(Venkatesan, 2014).For *Pf*mdr1184Finsymptomatic harbored 68.75%and asymptomatic 52% mutations, while *Pf*mdr1 D1246 had 6% mutants in both symptomatic and asymptomatic infections. Artemether-lumefantrine is used as firstline treatment in Kenya but single nucleotide polymorphisms in the *Pf*mdr1 gene may compromise its sensitivity because N86, 184F and D1246 are favoured by treatment with this drug(Okell, Reiter, Ebbe, Baraka, Bisanzio, Watson, Bennett, Verity, Gething, Roper, *et al*., 2018).A steady decrease of *Pf*mdr1codon 86 mutation was observed in Kenya following the drug policy change from SP to AL(Eyase *et al*., 2013).The observed frequencies on *Pf*mdr1 gene in the present data may be due to use of AL over time in Kombewathus imposing selective pressure similar to a previous studies in Kisumu (Eyase *et al*., 2013; Hemming-Schroeder *et al*., 2018).

The current study reported a frequency of symptomatic 47.05% and asymptomatic 37.93% respectively, for polymorphisms at *Pf*mrp1 codon I876V which was higher than polymorphisms at codon F1390I symptomatic 6.67% and asymptomatic 6.9%. These mutations were previously found to be linked with *in-vitro* parasite susceptibility and *in-vivo* response to ACT (Dahlström *et al*., 2009)and have been also reported in Kenyasimilar to the current study.Moreover, previous study in Myanmar region also reported frequencies of 58.8% and 8.5% for *Pf*mrp1 876V and 1390I respectively(Veiga *et al*., 2011). Only one mutant was observed in asymptomatic *Pf*mrp1 437A. The observed high frequency of 876V mutations which is similar to the current findings might be linked to AL drug pressure and subsequent selection and increased prevalence of *Pf*mrp1 I876V single nucleotide polymorphisms. Findings from the current study indicated that *P.falciparum* isolates from symptomatic and asymptomatic infections in Kombewa harbored some of the SNPs associated with antimalarial resistance for *Pf*mdr1 &*Pf*mrp1 genes that have been implicated. Although there was no significant variation of the SNPS in*Pf*mdr1 &*Pf*mrp1,

establishing the frequency of these drug resistance markers for timely detection and curbing the spread of mutations is key as ACT is used for treatment in Kombewa region specifically AL in the effort towards malaria elimination.

Sulphadoxine pyrimethamine is used in Kenya and Tanzania as intermittent preventive therapy during pregnancy(Mikomangwa *et al*., 2020). High frequency of mutants revealed in *Pf*dhfr C59Rwhich had 88.24% and 90.91% mutations for symptomatic and asymptomatic infections respectively in the current study is similar to a study on migrant workers from Africa reported in China(Yan *et al*., 2021)and another study on pregnant women in coastal Kenya(Gikunju *et al*., 2020)may suggest sustained resistance to sulphadoxine pyrimethamine in Kombewa region, which supports its use for intermittent prophylaxis treatment amongst pregnant women in this high malaria endemic lake region.The *Pf*dhfr I164L which is also linked to mutation in cycloguanil had one and two mutations for symptomatic and asymptomatic respectively, and has been reported withlowfrequencies in East Africa and Asia (Basuki *et al*., 2018; Lynch *et al*., 2017) but very rare in central Africa.It was also noted that the travellers study in China on migrant workers did not find any polymorphisms in *Pf*dhfr I164L and this maybe be attributed to high fitness cost(Yan *et al*., 2021).Furthermore, the absence of this mutation in Kisumu previously reported by Spalding and co-wokers indicates that theparasites habouring this mutation did not become widespread (Spalding *et al*., 2010).Taken together, these findings are harmonious with the evidence that I164L mutations are very rare Kenya and sub-Saharan Africa.The remaining codons *Pf*dhfr 16 and 22 did not have any mutations similar to Kenyan study conducted on samples from different sites including Kombewa(Juma *et al*., 2014).

Resistance to sulfadoxine has also been associated with single nucleotide polymorphisms in *Pf*dhps gene at codons S436A/F, G437A, K540E, A581G, and A613T/S (Bwire *et al*., 2020).Mutations at codons 437 and 540 were strongly associated with sulphadoxine pyrimethamine treatment failure, whereas the 436, 581, and 613 mutations confer some resistance.The current study analyzed *Pf*dhps codons S436A/F, G437A, A581G and no mutation was reported in both symptomatic and asymptomatic population suggesting that SP may still be used for prophylaxis during pregnancy. While studies have reported fixation of sulphadoxine pyrimrthamine resistant allele in Western Kenya (Spalding *et al*., 2010), and South East Asia (Alam *et al*., 2011), the contrary has been reported in Peru with decline in these alleles being

reported five years following change of policy(Vinayak *et al*., 2010).Fixation of the mutant alleles reported in the current study and others may be attributed to the fact that the fitness cost does not impact the population of the parasite(Alam *et al*., 2011). In addition, there might be the probability of additional selection pressure resulting from other antifolate drugs such as cotrimoxazole (Garner & Gülmezoglu, 2006; Gasasira *et al*., 2010).However, rapid spread of *P. falciparum* parasites that are resistant to SP in areas of malaria endemicity poses a major threat to the prevention of malaria in pregnancy (Hommerich *et al*., 2007; Mockenhaupt *et al*., 2007).The findings from the current study similar to previous study in this region that reported increased frequency of these polymorphisms (Hemming-Schroeder *et al*., 2018)have highlighted the variations in the alleles hence the need for continued surveillance.

Mutations in *Pf*crt codons 72-76 are known to confer resistance to previously chloroquine and other quinoline drugs including lumefantrine and amodiaquine (Holmgren *et al*., 2006; Sisowath *et al*., 2009). Moreover,*Pf*crt codons72, 76, & 356 did not have any mutations for either symptomatic or asymptomatic.The absence of mutations in codon 76 as shown in the current study indicates the conversion of the parasite population to the chloroquine sensitive alleles attributed to the comprehensive use of AL in Kenya for treatment of malaria and the reduction of amodiaquine use lead to the release of drug resistance pressure on *Pf*crt gene(Eyase *et al*., 2013).Mutations on *Pf*crt codon 356 were repoted in Uganda in 2.4% of the isolates in mixed alleles infection before use of AL but was not detected in the isolates after use of AL(Balikagala *et al*., 2017). The *Pf*crt codon 356 mutations were found in isolates collected from different parts of Africa from malaria patients in France who had travelled to sub saharan Africa 54.7%(Foguim *et al*., 2020), Malaysia 24% (Atroosh *et al*., 2012), Thailand 99.2% and Cambodia 67.7% (Dhingra *et al*., 2019).Increasing trends of wild type codon 76 have been reported in other areas of East Africa however the travellers study revealed mutations on *Pf*crt codon 76 was at 20.6% with significant associations in the frequencies of *Pf*crt codons 76 and 356 in West African isolates while no mutation was reported *Pf*crt codon 72 (Foguim *et al*., 2020) similar to the current study. *Pf*crt 326 & 371 had 3.23% (1/31) and 11.11% (4/36) mutations in asymptomatic only similar to a Nigerian study and is reported to be rare for *Pf*crt 371(Idowu *etal*., 2019). Studies have reported the potential selection of *Pf*crt K76 and *Pf*mdr1 N86/Y184/D1246 following treatment with AL (Happi *et al*., 2009; Sisowath *et al*., 2009) with reports of higher frequencies of these alleles than previously reported(Happi *et al*., 2009).The *Pf*crt gene had the

least number of mutations, and these may be attributed to the absence of drug pressure causing the parasites to revert to the wild form. This is a positive indicator that chloroquine may be re-introduced into the market.

The observed variability of single nucleotide polymorphisms trends in the current study are not novel, however Kombewa region supports the use of ACTs and SP for malaria infection treatment and prophylaxis respectively. This emphasizes the need for continous molecular surveillance of these strains particularly those harbouring mutations with a previously demonstrated association with antimalaria drug resistance as they have implications for planning strategies to cycle drugs or use multiple first-line therapies to maintain drug efficacy.

5.3 Variability of Gametocytes in Symptomatic and Asymptomatic Infections

The assessment of gametocyte variabilitywas assayed using *Pfs*16 and *Pfs*25 gene markers among the symptomatic and asymptomatic infections. In the current study the symptomatic exhibited slightly higher prevalence of gametocytes than asymptomatic, this may be attributed to the fact that gametocyte production is influenced by immunity among other factors (Price *et al*., 1999).Although the current study revealed that the gametocyte carriage was slightly higher in symptomatic than asymptomatic similar to previous studies reported in Kenya(Idris *et al*., 2016), the presence of gametocytes in asymptomatic population indicates that theymay serve as the infectious reservoirs of transmission in Kombewa as reported previously (Andagalu *et al*., 2022; Koepfli *et al*., 2021; WHO, 2018).This may be attributed to the fact that gametocyte densities remain modest relative to asexual densities so that transmissible forms of the parasite are masked from nonspecific immune responses by asexual forms and to avoid eliciting specific transmission-blocking immune responses (Mideo & Day, 2008; Taylor *et al*., 1997).

It is important to note that as drug resistance seizes a population, there occurs delays in the time taken to clear the peripheral parasitemia and this was significantly associated with subsequent gametocyte production therefore increased gametocyte carriage may be an essential factor that drives resistance of *P. falciparum*(White, 1999). A study reported that the parasite status i.e wild type or drug resistant parasite was significant in determining the conversion rates of gametocytes, moreover the conversion rates were not altered in response to drug in the case of drug resistant parasites(Reece *et al*., 2010). The study in Sudan by by Reece *et al* showed wild type parasites that are drug sensitive tend to transmit gametocytes at a slower rate following

exposure than the parasites habouring drug resistant genes promoting transmission and expansion of drug resistant parasites. Therefore this warrants the assessment ofgametocyte varibility and transmission in both symptomatic and asymptomatic infections.

The aim of this study was toassess prevalence of *Plasmodium* species, antimalarial drug resistance markers and gametocytes among the symptomatic and asymptomatic infections at Kombewa which is a holoendemic area. With recent declines in clinical symptomatic malaria, there is increasing evidence to suggest that asymptomatic parasite carriers play an important role in maintaining transmission (Iwagami *et al*., 2017; Lover, Baird, *et al*., 2018; Lover, Dantzer, *etal*., 2018; Niang *et al*., 2017; Sáenz *et al*., 2017). Asymptomatic infections play an essential role in continuity of malaria transmission and in clinical malaria (Niang *et al*., 2021). Critically, both infections may harbor varying antimalaria drug resistance genes(Tukwasibwe *et al*., 2014), hence enabling the expansion of mutant parasites that are drug resistant (Nyunt, Shein, *et al*., 2017; Nyunt, Wang, *et al*., 2017) yet very few studies have been done to compare the symptomatic and asymptomatic infections.

5.4 Limitations of the Current Study

Samples were collected from one geographic region with one transmission setting. Data from different regions with varying malaria endemicities preferably would give a much better assessment of symptomatic and asymptomatic infections. Study period stretches through the years that COVID 19 pandemic was rife however implication of the pandemic on malaria infections was not assessed in this study.

CHAPTER SIX

SUMMARY OF FINDINGS, CONCLUSION AND RECOMMENDATION

6.1 Summary of Findings

In the efforts towards malaria control and elimination, the accurate diagnosis and timely treatment of both symptomatic and asymptomatic *Plasmodium* infections is essential since they entail *Plasmodium* species, drug resistance markers and gametocytes. The current study has highlighted the variability of these parameters hence the need for continued surveillance and use of sensitive technology to screen and ascertain both symptomatic and asymptomatic infections. Moreover, their role in malaria transmission needs more in-depth analysis tostop and limit the spread of antimalaria drug resistance and transmission of malaria. Due to their elusive nature, these infections have significant implications for the development and implementation of anti-malarial measures.

6.2 Conclusions

i. Four *Plasmodium* species were detected, and theirprevalence was variable in both symptomatic and asymptomatic infections. The co-infections/mixed species may interact depending on the species present hence more studies on the same are needed.

ii. The frequencies ofsingle nucleotide polymorphisms in *Pf*mdr1, *Pf*mrp1, *Pf*dhfr &*Pf*crt genes observedin symptomatic and asymptomatic infections may have implications such as delayed parasite clearance and subsequent treatment failure because Kombewa supports the use of ACTs and SP for malaria infection treatment and prophylaxis.

iii. Although the variability in gametocyte carriage was marginal in symptomatic and asymptomatic infections,presence of gametocytes in asymptomatic population may indicate that they serve as the silent infectious reservoirs of transmission in Kombewa hence the sustained high malaria transmission.

6.3 Recommendations from the Current Study

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

i. The*Plasmodium* species prevalencewas marginallyvariable in symptomatic and asymptomatic hence the need to monitor their trends in both infections because they are linked to fatal consequences even in well resourced settings.

ii. Mutations revealed in this study highlight the need for molecular surveillance of these SNPS associated with antimalaria drug resistance for planning strategies because Kombewa supports the use of AL and SP as therapeutics. This is essential for timely intervention strategies such as cycling drugs or use of multiple first-line therapies to maintain drug efficacy and curb the spread of resistance genes insymptomatic and symptomatic infections.

iii. The marginal variability in gametocyte carriage in symptomatic and asymptomatic infections suggeststhat malaria intervention strategies should not only targetsymptomatic infections. Asymptomatic infections should also be consideredin the malaria elimination programs by putting in place proper structures with the help of the community at the local and national level for screening and treating to reduce, and ultimately prevent malaria transmission.

6.4 Recommendations for Future Studies

Based on the current study findings, future studies should consider the following:

i. To increase the chances of success in eliminating malaria, strategies for control should be reoriented from targeting individual species sequentially which is currently influenced by *P.falciparum,* to a strategy that is inclusive of all species simultaneously. It is important to integrate molecular detection tools into all epidemiological studies, as a significant proportion of asymptomatic submicroscopic malaria infections are missed by conventional diagnostic methods. Therefore, to better understand the dynamics and transmissibility of all *Plasmodium* species in symptomatic and asymptomatic infections, it is crucial to study all species.

ii. Surveillance for genetic polymorphisms associated with antimalarial resistance at greater spatiotemporal scale will present an opportunity for broad characterization of parasite genotypes in both symptomatic and asymptomatic malaria infections. Molecular epidemiology of these drug resistance genes is key as ACT is used for treatment in Kenya specifically AL in the effort towards malaria elimination.

iii. Given the high prevalence of gametocytes carriage , mathematical modelling may be key in determining the role of both symptomatic and asymptomatic parasite carriers in the community. Targeting both populations will have a considerably larger impact on preventing transmission than those interventions that target symptomatic cases only.

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APPENDICES

Appendix 1: Research Approval

DEPARTMENT OF THE ARMY
WALTER REED ARMY INSTITUTE OF RESEARCH
503 ROBERT GRANT AVENUE
SILVER SPRING, MD 20910-7500

REPLY TO
ATTENTION OF

FCMR-UWS-HP

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

SUBJECT: Continuing Review Report Approval for the Minimal Risk Human Subjects Research Protocol, **WRAIR #2454**, OHRO Log #E04188.a-c

1. The continuing review report, dated 5 October 2023 (corrected 9 November 2023), for the protocol **WRAIR #2454**, OHRO Log #E04188.a-c, titled, "Epidemiology of Malaria and Drug Sensitivity Patterns in Kenya," (Version 2.8, dated 15 September 2022) and supporting documents, submitted by Hosea Akala, PhD, Kenya Medical Research Institute (KEMRI)/Walter Reed Project (WRP) is approved.

2. The continuing review report covers the reporting period from 1 October 2022 through 30 September 2023. This study is currently enrolling subjects.

3. As this is a minimal risk protocol, the continuing review report was reviewed and approved via expedited review procedures according to 32 CFR 219.110(b)(1)(i) and 45 CFR 46.110(b)(1)(i), per expedited category 9, continuing review of research, not conducted under an investigational new drug application or investigational device exemption where categories two (2) through eight (8) do not apply but the Institutional Review Board (IRB) has determined and documented at a convened meeting that the research involves no greater than minimal risk and no additional risks have been identified. This study continues to meet the requirements under 32 CFR 219.111, 45 CFR 46.111, and 45 CFR 46.404, as it involves children participating in a not greater than minimal risk study.

Though pregnant women, or women who become pregnant may participate in the study, according to the DoDI 3216.02 (June 2022), 45 CFR 46.204 does not apply as this is a minimal risk protocol.

The protocol will continue to require at least annual continuing review, per 32 CFR 219.109(e), as this protocol was approved in accordance with the Pre-2018 Common Rule. Please note that this protocol could potentially meet the criteria to be governed under the 2018 Common Rule, which allows for submission of a progress report rather than a continuing review report. If interested in operating under the rule changes that took effect on 21 January 2019, please contact the HSPB Point of Contact (POC) as the protocol and supporting documents will need to be amended. Amended documents will need to undergo subsequent WRAIR IRB and local IRB review and approval.

4. The KEMRI Scientific and Ethics Review Unit (SERU) approved this protocol for continuation until 6 May 2024, per the memorandum dated 26 April 2023.

To avoid an interruption in work, please provide a copy of the updated ethics board approvals to the WRAIR HSPB as they become available.

In Search of Better Health

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KEMRI/RD/22 **April 26, 2023**

TO: **DR. HOSEA AKALA**
PRINCIPAL INVESTIGATOR

THROUGH: **THE DEPUTY DIRECTOR, CCR,**
NAIROBI

Dear Sir,

RE: **SERU PROTOCOL NO. 3628 (*REQUEST FOR ANNUAL RENEWAL*): EPIDEMIOLOGY OF MALARIA AND DRUG SENSITIVITY PATTERNS IN KENYA**

Thank you for the continuing review report for the period **29th February 2022 to 28th February 2023**.

This is to inform you that the expedited review team of the KEMRI Scientific and Ethics Review Unit (SERU) conducted the annual review of the above referenced application and was of the informed opinion that the progress made during the reported period is satisfactory. The study has therefore been granted **approval**.

This approval is valid from **May 07, 2023** for a period of **one (1) year**. Please note that authorization to conduct this study will automatically expire on **May 06, 2024.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **March 25, 2024**.

You are required to submit any amendments to this protocol and any other information pertinent to human participation in this study to the SERU for review prior to initiation.

You may continue with the study.

Yours faithfully,

ENOCK KEBENEI,
THE ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT.

DEPARTMENT OF THE ARMY
WALTER REED ARMY INSTITUTE OF RESEARCH
503 ROBERT GRANT AVENUE
SILVER SPRING, MD 20910-7500

REPLY TO
ATTENTION OF

FCMR-UWS-HP

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

SUBJECT: Continuing Review Report Approval for the Greater than Minimal Risk Human Subjects Research Protocol, **WRAIR #2739**, OHRO Log #E04203.a-c

1. The corrected continuing review report, dated 20 April 2023, for the protocol **WRAIR #2739**, OHRO Log #E04203.a-c, titled, "Clinical Investigation Study to Evaluate the Consistency and Reproducibility of Two Consecutive Mosquito Feeding Assays in Adults with Varying *Plasmodium falciparum* Gametocyte Densities," (Protocol Version 1.4, dated 31 October 2022) and supporting documents, submitted by Hoseah Akala, PhD, Kenya Medical Research Institute (KEMRI)/Walter Reed Project (WRP), Kenya is approved.

2. The continuing review report covers the reporting period from 30 March 2022 through 29 March 2023. This study is closed to enrollment, remains open for the analysis of specimens and data and is awaiting final closure by the Sponsor.

3. As this greater than minimal risk protocol is permanently closed to enrollment and remains open for the analysis of specimens and data, the continuing review report was reviewed and approved via expedited review procedures according to 32 CFR 219.110(b)(1)(i), and 45 CFR 46.110(b)(1)(i), per expedited category 8c, continuing review of research previously approved by the convened IRB where the remaining research activities are limited to data analysis. This study continues to meet the requirements under 32 CFR 219.111 and 45 CFR 46.111.

4. The KEMRI Scientific and Ethics Review Unit (SERU) approved this protocol for continuation until 22 September 2023, per the memorandum dated 23 September 2022.

An Institutional Agreement for Institutional Review Board (IRB) Review (IAIR) between the KEMRI SERU and PATH was established for this study on 25 November 2020, allowing PATH to rely on the KEMRI SERU for ethical review.

To avoid an interruption in work, please provide a copy of the updated ethics board approvals to the WRAIR HSPB as they become available.

5. This review also constitutes the Human Research Protections Official (HRPO) continuing review approval for the participating non-DoD collaborators in accordance with DoDI 3216.02, Section 3.6, (b)5. This HRPO review applies only to this continuing review action; all other reporting and review requirements by the US Army Medical Research and Development Command (USAMRDC), Office of Human and Animal Research Oversight (OHARO), Office of Human Research Oversight (OHRO), remain in effect.

6. This study continues to be sponsored and funded by PATH with support from the Bill and Melinda Gates Foundation.

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KEMRI/RD/22 **September 19, 2023**

TO: **DR. HOSEAH AKALA,**
PRINCIPAL INVESTIGATOR.

THROUGH: **THE DEPUTY DIRECTOR, CCR,**
NAIROBI.

Dear Sir,

RE: **PROTOCOL NO. SERU 4082 (*REQUEST FOR ANNUAL RENEWAL*): CLINICAL INVESTIGATION STUDY TO EVALUATE THE CONSISTENCY AND REPRODUCIBILITY OF TWO CONSECUTIVE MOSQUITO FEEDING ASSAYS IN ADULTS WITH VARYING PLASMODIUM FALCIPARUM GAMETOCYTE DENSITIES.**

Thank you for the continuing review report for the period **14 July 2022 to 13 July 2023**

This is to inform you that the expedited review team of the KEMRI Scientific and Ethics Review Unit (SERU) conducted the annual review of the above referenced application and was of the informed opinion that the progress made during the reported period is satisfactory. The study has therefore been **granted approval**.

This approval is valid from, **September 23, 2023** through to **September 22, 2024**. Please note that authorization to conduct this study will automatically expire on **September 22, 2024.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **August 11, 2024.**

You are required to submit any amendments to this protocol and any other information pertinent to human participation in this study to the SERU for review prior to initiation. You may continue with the study.

Yours faithfully,

ENOCK KEBENEI,
THE ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

Appendix 2: Research Approval

REPUBLIC OF KENYA

NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Ref No: **891801**

Date of Issue: **27/September/2023**

RESEARCH LICENSE

This is to Certify that Ms.. Gladys Chebet Chemwor of Maseno University, has been licensed to conduct research as per the provision of the Science, Technology and Innovation Act, 2013 (Rev.2014) in Kisumu on the topic: The Role of Plasmodium Species Composition and Drug Resistance Genes in Symptomatic and Asymptomatic Malaria Transmission in Kombewa, Kenya for the period ending : 27/September/2024.

License No: **NACOSTI/P/23/29508**

891801

Applicant Identification Number

Director General
NATIONAL COMMISSION FOR SCIENCE,TECHNOLOGY & INNOVATION

Verification QR Code

NOTE: This is a computer generated License. To verify the authenticity of this document, Scan the QR Code using QR scanner application.

See overleaf for conditions

Appendix 3: Consent Form

Adult Consent Form (includes parental or legal guardian consent) for MoH sites

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

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

PRINCIPAL INVESTIGATOR: Dr. Hoseah Akala, Ph.D.

1 INTRODUCTION

You (your child) are being asked to participate in this research study. Participation in this study is voluntary. Refusal to participate will involve no penalty or loss of benefits to which you (your child) are otherwise entitled. You (your child) may discontinue participation at any time without penalty or loss of benefits.

This research study is supported by the United States Department of Defense. Funding for this study comes from the Global Emerging Infections Surveillance and Response System (GEIS), Armed Forces Health Surveillance Center. This study is a continuation of a study that has been running since 2007 that has helped describe how the malaria germs in different parts of Kenya respond to the drugs that are used to treat malaria.

2 PURPOSE OF THE STUDY

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

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

3 WHO CAN PARTICIPATE IN THIS STUDY

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

4 WHO CANNOT PARTICIPATE IN THIS STUDY

You (your child) cannot participate in this study if

1. You (your child) are unwilling to give blood.
2. You are not capable of giving informed consent.
3. You (your child) are currently detained by the Kenya Government Department of Correctional Services.

4. You (your child) were previously enrolled in the study this year.
5. Your child who intends to get enrolled weighs less than 5 kg.

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

5 APPROXIMATE NUMBER OF VOLUNTEERS TAKING PART IN THIS STUDY

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

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

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

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

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

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

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

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

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

7 POTENTIAL RISKS AND DISCOMFORTS

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

8 ANTICIPATED BENEFITS TO VOLUNTEERS

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

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

9 ALTERNATIVE TO PARTICIPATION

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

10 PAYMENT FOR PARTICIPATION

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

For volunteers recruited from the MoH sites, support for public transportation to return to the study site for each follow up visit, and return home, will be provided to you (your child). The amount will be 500 Kenyan shillings per visit. Support for public transportation will also be provided for unplanned visits that are directly related to participation in this study.
Volunteers from the KDF sites are not expected to use public transport, and as such there will not be any direct compensation for transportation.
Other than medical care that may be provided and any other payment specifically stated in the consent form, there is no other compensation available for your participation in this research.

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

Your (your child's) blood samples will be stored and used only for the tests associated with this study. However, you (your child) may grant permission for the malaria germs in your blood to be used for other studies in the future, some of which may be done outside the country. The laboratories that will be used include the Walter Reed Army Institute of Research Labs in the US together with its overseas labs located in Thailand and Peru, and Oxford University Sanger Institute labs in the United Kingdom You (your child) will be given a separate form to fill out which will allow you (your child) to say whether you (your child) will or will not allow your (your child's) blood samples to be used for future studies. The stored malaria germs for future studies will not have any items that could identify you as the original source, such as your name. No genetic testing will ever be done. There is no possibility that your samples could be used for developing a commercial product.

12 DURATION OF PARTICIPATION

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

13 ASSURANCE OF CONFIDENTIALITY

Records relating to your (your child's) participation in the study will remain confidential to the extent possible. Research records will be kept in a locked file at Walter Reed Project, Kisumu, Kenya. Your (your child's) name will not be used in any report resulting from this study. All computerized records and laboratory specimens will contain only a unique study number for you (your child), not your (your child's) name, or any other personal identifying information. Computer records will be password protected and accessed by

authorized study personnel only. Research records will be kept until all data analyses are completed.

14 PARTICIPATION AND WITHDRAWAL

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

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

15 WITHDRAWAL OF PARTICIPATION BY THE INVESTIGATOR

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

You (your child) may also be removed from this study without consent if:

a) you (your child) do not follow the study procedures
b) in the opinion of the study physicians, it is in your (your child's) best interest,

16 NEW FINDINGS

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

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

17 REVIEW OF RESEARCH RECORDS

It should be noted that representatives of the US Army Medical Research and Materiel Command and KEMRI are eligible to review research records as a part of their responsibility to protect human subjects in research. The research records will be made

available only to investigators and clinical hospital personnel who may need this information to treat you (your child), or to members of the Ministry of Health who require this information for administrative reasons.

18 PERSONS TO CONTACT FOR ANSWERS TO RESEARCH RELATED QUESTIONS

If you think you (your child) have a medical problem related to this study, you may report this to Principal Investigator of the Study - Dr. Hoseah Akala, Malaria Drug Resistance Laboratory, United States Army Medical Research Directorate/KEMRI (The Walter Reed Project), Kisumu, Kenya, Tel: +254722329845 or +254202023858.

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

If during the course of this study, you have questions concerning the nature of the research, you should contact the Principal Investigator of the Study - Dr. Hoseah Akala, at the Walter Reed Project, telephone +254722329845 or +254202023858. If you are not satisfied, you may also contact the Secretary of the Scientific and Ethics Review Unit, c/o Kenya Medical Research Institute, P.O. Box 54840, Nairobi, Kenya, tel. 020-272-251 or 0722205901. The Scientific and Ethics Review Unit of Kenya Medical Research Institute is a body that is independent of the study team.

20 RIGHTS OF RESEARCH SUBJECTS

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

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

If Subject is a minor Name of Subject: Printed name of participant/parent/guardian:

Signature of participant/parent/guardian:

Date:

WITNESS: I have witnessed the explanation of the research study to the participant/parent/guardian. The participant was given an opportunity
to ask questions, and the participant's questions, if any, were answered. Printed name of witness:

Signature of witness:

Date:

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

Printed name of individual obtaining consent:

Title:

Signature: Date:

Printed name:

Signature: Date:

Consent for Testing for Blood Borne Infections, Future Research Use and Long-Term Blood Sample Storage
Adult Consent Form (includes parental or legal guardian consent)

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

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

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

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

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

- NO. I do not want my (my child's) samples to be stored for future use If Subject is a minor Name of Subject: Printed name of participant/parent/guardian:
Signature of participant/parent/guardian:

Date:

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

Printed name of witness:

Signature of witness: Date:

Printed name of individual obtaining consent for long term storage:

Title:

Signature: Date:

CONSENT FOR TESTING FOR BLOOD BORNE INFECTIONS:
By signing this form, you AGREE that you have read the information provided above, or that it has been explained to you. You have also been given an opportunity to ask questions and these have been answered to your satisfaction. A copy of this form will be given to you (your child). Sign YES if you agree and NO if do not agree to allow testing for blood borne infections.

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

- NO. I do not want my (my child's) samples to be tested for blood borne infections If Subject is a minor Name of Subject:
Printed name of participant/parent/guardian:

Signature of participant/parent/guardian:

Date:

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

Printed name of witness:

Signature of witness: Date:

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

Title:

Signature: Date:

Printed name:

Signature: Date:

Appendix 4: Assent form

Agreement to Participate in a Research Project
Assent Form, Ages 13 through 17 Years

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

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

PRINCIPAL INVESTIGATOR: Dr. Ben Andagalu, MD MSc

1. INTRODUCTION

We are inviting you to be in this research study. A research study is something like a science project you do in school. This research study is supported by the United States Department of Defense.

Before you decide whether to volunteer or not to volunteer, you must understand the purpose, how it may help you, what risks are there, and what is expected of you. Once you understand the study, and if you agree to volunteer, you will be asked to sign this form. You can only be in this study if your parent (s) or legal guardian also agree.

This form gives you information about the study. A member of the study team will talk to you about the study and answer any questions you have. We will ask you to sign this form to show that you understand the study. We will give you a copy of this form to keep. It is important that you know:

- You do not have to join this study. Your participation is entirely up to you. If you do not want to participate, you will not be pushed, punished or lose any medical treatment.
- You may change your mind and drop out of the study any time you want.
- If we make any important changes in the study, we will tell you about it and will ask you if you still want to be in the study.

After you read the explanation, you should ask any questions needed for you to understand better what the study is about.

2. PURPOSE OF THE STUDY

a. What is the research study about?
Your doctor thinks you may have malaria. This means that you might be having germs that cause malaria in your blood. This project you are being asked to participate in is to

learn about malaria germs and what drugs best kill the malaria germs found in this part of Kenya.

b. Why are you being asked to participate?
We are asking you because your illness today fits what we are looking for.

c. How many people will participate?
Up to 100 total volunteers per week, from all of the Ministry of Health sites we are collecting from, as well as 2500 individuals every year from the Kenya Defence Forces sites.

3. PROCEDURES

What will happen to you if you agree to participate?
Today, we will ask you some questions about where you live and medications you have taken and take a blood sample from your arm. We will take about 1 teaspoon (5 milliliters) of blood from your arm vein. The whole process will take about 1 hour. When we draw blood from your arm, we will try only once. If we cannot get blood on the first try, we will stop. A clinic doctor will provide medicine to cure you of malaria germs if the test shows you have malaria. We will send your blood to the laboratory and test what medicines will kill the malaria germs. This is the experimental part of what we are asking of you.

In 7 days, we ask that you come back to see us. We will ask you some questions only about how you responded to the medication you took, and obtain a blood sample (5 ml or about 1 teaspoon) from your arm. The doctor will give you other medications that will cure you.. This will take about 1 hour.

If you are in a special group and agree to come back 5 additional times, on days 2, 3, 14, 28 and 42, at each visit, we will ask some questions only about how you responded to the medication you took, and obtain a drop of blood from your fingertip to see if you still have the malaria germs have gone away. We will tell you the results. If you still have malaria, we will ask you for a blood sample (5 ml or about 1 teaspoon) from your arm. If you do not have malaria, no further blood will be taken. This will take about 1 hour.

If you agree, some of your blood may be kept at the main laboratory for a longer period for further testing in the future. Some of these tests may be performed at laboratories outside the country. If you agree, your blood will also be tested for a group of infections called blood borne infections. You can choose whether or not you want to have your blood kept for a longer period and whether or not the test for blood borne infections will be done – that will not affect your participation in this study. Your parent/guardian will be given a separate form to record your decision and sign.

We will ask you to stop being in this study if you have any bad side effects or your doctor thinks it is best for you to stop participating in this study.

You can participate in this study several times, as long as it is not in the same year. You will be asked to sign a new form each time you are enrolled.

4. POTENTIAL RISKS, DISCOMFORT, OR PAIN

The blood draw from your arm usually takes about 2 minutes, and can cause some slight pain when the needle is inserted. Having your blood drawn may hurt a little. You may also feel dizzy or you may faint when blood is drawn. You may have some bleeding or a bruise after the blood is drawn. For the fingertip blood sample, we will use a device that minimizes pain. The fingerstick causes slight pain for several seconds.

5. BENEFITS

We will test your blood when you come back to see if malaria germs are gone and to check if the treatment you were given has worked and you will be told the results.
If our test shows that the medicine you received did not work, you will be able to see a doctor to receive more medicine.

If you let us study the germs in your blood, it may help other children, because we may know what medicines are best to make them feel better.

6. ALTERNATIVE TO PARTICIPATION

The only alternative to participation in this research is to not participate.

7. CONFIDENTIALITY

We will keep the records of this study private to the extent possible. We will not tell anyone you are in the study. Only the people working on the study will know your name. They will keep this information only in case we have to find you later for medical reasons.

All of the data concerning you collected during the study will be identified by numbers. All records for future use or review will also be identified only with your volunteer number. The records will be kept until all analyses are completed.

8. COSTS AND COMPENSATION

You or your parent will not be asked to pay any money for you to participate in this study. You will not receive money for participating. The study provide support for public transportation for you and your parent(s)/guardian from home to the study site and return, at 500 Kenyan shillings per visit.

9. CONSEQUENCES OF WITHDRAWING

You may stop being in the study at any time, and nothing bad will happen to you or your parent(s)/guardian.
Your parent will be asked for permission to store your blood samples that contain malaria parasites indefinitely for possible use in other research studies.

ASSENT. By signing this form, you agree that you have read the information provided above, or had it explained to you, talked to your doctor or a member of the study team about the study and understand it, and want to be in the study. You have been given an opportunity to ask questions and all have been answered to your satisfaction. You also agree that we have talked to you about the risks and benefits of the study, and about other choices. You may drop out of the study at any time, and no one will mind and nothing will change about your medical care. You may call Principal Investigator of the Study - Dr. Ben Andagalu, Malaria Drug Resistance Laboratory, United States Army Medical Research Directorate/KEMRI (The Walter Reed Project), Kisumu, Kenya at telephone +254716004851 or +254202023858 if you have any questions.

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

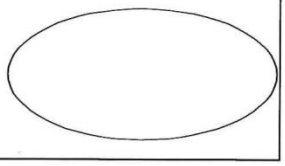
A signed copy of this form will be given to you and your parent/guardian.

Printed name of participant: _____

Signature of participant: _____

Date: _____

Thumbprint of subject if unable to sign:



WITNESS: I have witnessed the explanation of the research study to the participant. The participant was given an opportunity to ask questions, and the participant's questions, if any, were answered.

Printed name of witness: _____

Signature of witness: _____

Date: _____

INDIVIDUAL OBTAINING ASSENT: I certify that I have explained to the above individual the nature and purpose of this study, potential benefits, and possible risks associated with participation in this study. I have answered all questions that have been raised.

Printed name of individual obtaining consent: _____

Title: _____ Date: _____

Signature: _____

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

Thumbprint if unable to sign

-_____ **YES**. I will allow my samples to be stored for future use

-_____ **NO**. I do not want my samples to be stored for future use

ASSENT FOR TESTING FOR BLOOD BORNE INFECTIONS:
By signing this form, you **AGREE** that you have read the information provided above, or that it has been explained to you. You have also been given an opportunity to ask questions and these have been answered to your satisfaction. A copy of this form will be given to you and your parent/guardian.
Sign **YES if you agree** and **NO** if **do not agree** to allow testing for blood borne infections.

-_____ **YES**. I will allow my samples to be tested for blood borne infections

-_____ **NO**. I do not want my samples to be tested for blood borne infections

Thumbprint if unable to sign

Date: _____

WITNESS: I have witnessed the explanation regarding testing for storage of samples as well as testing for blood borne infections to the participant. The participant was given an opportunity to ask questions, and the participant's questions, if any, were answered.

Printed name of witness: _____

Signature of witness:_____ Date:_____

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

_____

Title: _____

Signature:_____ Date: _____

INVESTIGATOR

Printed name: _____

Signature: _____

Date: _____