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COLLEGE OF HEALTH SCIENCES

K13 PROPELLER GENE POLYMORPHISMS IN *PLASMODIUM FALCIPARUM* PARASITES ISOLATED FROM MALARIA SYMPTOMATIC PATIENTS ON ARTEMETHER-LUMEFANTRINE TREATMENT AT KASANGATI HEALTH CENTRE IV

BY

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DECLARATION

I Bitek Brian, hereby declare that the work in this research dissertation is entirely my own and that it has not been submitted to any other academic institution for any other academic award.

Signature..... **Date**.....15/08/2018.....

The dissertation has been submitted with the approval of the following supervisors

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DEDICATION

I dedicate this work to my late father, Mr. Otim Seth (RIP) and my mother, Mrs. Otim Evalyne.

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LIST OF ABBREVIATIONS

WHO	:	World Health Organization
K13	:	Kelch 13
ACT	:	Artemisisnin-based Combination Therapy
FP2	:	Falcipain 2
ARE	:	Antioxidant Response Element
Nrf2	:	Nuclear erythroid 2-related factor 2
PCR	:	Polymerase Chain Reaction
MARE	:	Maf Recognition Element
*Pf*PI3k	:	*P. falciparum* phosphatidylinositol-3- kinase
EDTA	:	Ethylenediaminetetraacetic Acid
TAE	:	Tris Acetate Ethylenediaminetetraacetic acid
GMS	:	Greater Mekong Sub region
BC	:	Before Christ
CDC	:	Centre for Disease Control
ADRs	:	Adverse Drug Reactions
Fe^{2+}	:	Ferrous iron
BTB/POZ	:	Broad-Complex, Tramtrack and Bric a brac/ POxvirus and Zinc finger
SERCA	:	Sarco/Endoplasmic Reticulum Ca^{2+}-ATPase
MAKCHS	:	Makerere University College of Health Sciences
Ca^{2+}	:	Calcium ions
KEAP1	:	Kelch Like Erythroid cell-derived Associated Protein 1

WBCs	:	White Blood Cells
SNP	:	Single Nucleotide Polymorphism
PBS	:	Phosphate Buffered Saline
dNTPs	:	Deoxynucleotide Triphosphates
ALT	:	Alanine Aminotransferase
UV	:	Ultra Violet
w/v	:	Weight/ Volume
v/v	:	Volume/ Volume
CYP	:	Cytochrome P450
*pf*MDR	:	*Plasmodium falciparum* multi drug resistance gene
rpm	:	revolutions per minute
BLAST	:	Basic Local Alignment Search Tool
NCBI	:	National Center for Biotechnology Information

OPERATIONAL DEFINITIONS

Nested PCR: This is a modification of standard PCR that involves two pairs of primers, the first set of primers amplifies a larger region within which the sequence of interest is contained while the second pair of primers amplifies the specific DNA sequence of interest (Snounou *et al*., 1993).

Primers: These are short single-stranded oligonucleotides which anneal to specific sequences in the template DNA and serve as an initiator for DNA synthesis.

Artemisinin resistance: Artemisinin resistance is defined as delayed parasite clearance following treatment with an artesunate monotherapy, or after treatment with an ACT (World Health Organization, 2015b).

ABSTRACT

Background: In 2003, World Health Organization (WHO) recommended the use of Artemisinin-based Combination Therapy (ACTs) for treatment of uncomplicated malaria and today ACTs form the backbone of malaria therapy. Development and spread of resistance to this class of drugs could be catastrophic in terms of malaria control and elimination efforts globally.

Main objective: To assess K13 propeller gene mutations among *Plasmodium falciparum* parasites isolated from malaria symptomatic patients on Artemether-Lumefantrine (AL) treatment at Kasangati Health Centre IV, Wakiso district.

Methods: This was a nested cross-sectional study in which *P. falciparum* malaria outpatients at Kasangati Health Center IV were purposively sampled and recruited into the study upon acquiring consent. A portion of capillary blood was collected from each patient and used to screen for *Plasmodium* infection using thick blood smears and thin blood smears to identify the species. Blood obtained from positive malaria patients was spotted on Whatman 903 filter papers. The patients were requested to report back to the hospital on day 7 to assess treatment success which was done using thick blood smears. *P. falciparum* DNA was extracted from the dried blood spots on the filter paper using Chelex resin method. The parasite DNA was then amplified using nested PCR method. The amplicons were sequenced using Sanger sequencing method. The sequences were then analyzed using MEGA 6.0 software.

Results: A total of 1466 out-patients were screened for *Plasmodium* infection of which, 6.9% (102/1466) were confirmed to have *P. falciparum* infection. The mean age (± SD) of the study participants was 24.7 ± 9.3 with the majority, 59% (59/100) being females. A total of 82 patients returned to the hospital on day 7 for follow-up assessment of parasite clearance after completing the dose of AL. No patient was found to be having *P. falciparum* parasites after treatment. *P. falciparum* DNA was successfully extracted from 70 blood samples and 67 amplicons were successfully sequenced. Mutations in the *P. falciparum* K13-propeller gene were detected in 2 of the 67 sequenced amplicons. Nonsynonymous mutations were detected at codon positions S522C and P667T in blood samples collected from different patients.

Conclusions: There is no resistance to artemisinin among the *P. falciparum* parasite population found in patients receiving medical treatment at Kasangati Health center.

CHAPTER ONE: INTRODUCTION

1.1 Background

The World Health Organization (WHO) estimated that about 214 million clinical cases of malaria with approximately half a million deaths occurred globally in 2015 (World Health Organisation, 2018b). Of all malaria cases globally, *Plasmodium falciparum* malaria is responsible for over 90% of the cases (Sinclair, Zani, Donegan, Olliaro, & Garner, 2009). Malaria poses a major social and economic burden that affects human development especially in endemic countries like Uganda. In these countries, malaria alone accounts for as much as 40% of public health expenditure and 30% to 50% of hospital admissions (Sinclair *et al*., 2009).

Sub-Saharan Africa continues to carry a disproportionately high share of the global malaria burden. In 2015, the region alone was home to about 76% of malaria cases and up to 75% deaths globally (World Heath Organisation, 2017). Uganda has the third highest incidence of malaria after the Democratic Republic of Congo and Nigeria in Sub-Saharan Africa (Uganda Ministry of Health, 2015). In Uganda, malaria is the commonest disease causing morbidity and mortality especially in children below five years (Kiwanuka, 2003). Hospital records suggest that malaria is responsible for 30 to 50 percent of outpatient visits, 15 to 20 percent of admissions, and 9 to 14 percent of inpatient deaths (Uganda Ministry of Health, 2015).

In 2003, the WHO recommended the use of Artemisinin-based Combination Therapy (ACTs) for treatment of uncomplicated malaria (World Health Organisation, 2016). This was majorly because the anti-malarial drugs at the time such as (Chloroquine and Sulfadoxine–Pyrimethamine (SP)) were no longer as effective due to high levels of *plasmodium* resistance. (Adhin, Labadie-Bracho, & Vreden, 2012). Over the years, ACTs have become the first line treatment for both complicated and uncomplicated *P. falciparum* malaria in nearly all endemic countries. Development and spread of resistance to these drugs could be very catastrophic in terms of malaria control and elimination efforts globally (Conrad *et al*., 2014). Uganda adopted the use of ACT drugs in 2004 and currently, they are the most commonly used medicines in malaria treatment and are also recommended as first-line agents for treatment of uncomplicated malaria (Bukirwa *et al*., 2006).

Recently, there have been reports of development of resistance to artemisinin and its derivatives in Southeast Asia especially around the Greater Mekong Sub-region (Ariey *et al*., 2014). According to WHO, artemisinin resistance is defined as delayed parasite clearance following treatment with artesunate monotherapy, or after treatment with an ACT (World Health Organization, 2015b). Several mutations in the Kelch 13 (K13) propeller gene of *P. falciparum* have been proven to be associated with delayed parasite clearance both *in vitro* and *in vivo* (Ariey *et al*., 2014). In Cambodia, detected mutations prevalent in this gene that were confirmed to confer resistance to artemisinins included (C580Y, R539T, and Y493H) (Ariey *et al*., 2014). This observation is a cause for worry as currently there are no alternative highly effective medicines for malaria treatment (Adhin *et al*., 2012).

In a study carried out in Uganda by Conrad *et al.,* (2014), mutant alleles in the K13 gene were detected among *P. falciparum* parasites isolated from children in Tororo district. However, the mutations detected in the K13 gene were not associated with delayed parasite clearance. In another study by Ocan *et al.,* (2016), mutant alleles were also detected in the K13 gene of *P. falciparum* parasites isolated from adult patients in northern Uganda. None of the mutations observed in these two studies were similar to those observed in Cambodia that were associated with delayed *Plasmodium* parasite clearance. These findings therefore, call for more regular monitoring of resistance development especially in sub-Saharan Africa, a region with the highest malaria burden globally (Conrad *et al*., 2014; Ocan *et al*., 2016).

Mutations conferring resistance to Lumefantrine have also been identified and confirmed by *in vitro* studies and they include N86, 184F, and D1246 within the *pf*mdr gene of the *P. falciparum* parasite (Malmberg *et al*., 2012). In Uganda, these mutations have been detected and shown to be on the increase among the *P. falciparum* population. This observation is consistent with the increased use of Artemether-Lumefantrine (A-L) as first-line treatment for uncomplicated malaria over the years (Taylor *et al*., 2017).

In the current study, *P. falciparum* parasites isolated from symptomatic malaria patients were screened for mutations in the K13 propeller gene. The association between the observed mutations and parasite clearance outcomes after treatment with A-L was also assessed.

1.2 Problem statement

To date ACTs constitute the back bone for malaria treatment and control globally. The recent discovery of resistant strains of *P. falciparum* to artemisinin agents in the Greater Mekong Sub region poses a threat to malaria management. Development and spread of *P. falciparum* resistance to artemisinin and its derivatives will limit malaria treatment options significantly. Limited information on independent molecular biomarkers of artemisinin resistance especially out-side of Southeast Asia greatly affects effective monitoring of resistance. This is because different *P. falciparum* K13-gene mutations whose role in artemisinin resistance is not known are being detected out-side Southeast Asia. It is thus not known to-date if there exists K13-gene mutations in *P. falciparum* parasite population in Sub-Saharan Africa that confers resistance to artemisinin agents. This therefore, calls for routine monitoring of molecular markers in the K13 propeller gene of *P. falciparum* and their effect on artemisinin agents especially in malaria endemic areas like Uganda. Artemisinins are usually given in combinations and A-L is the most common ACT administered in Uganda. Mutations conferring resistance to Lumefantrine (*pf*mdr1 N86, 184F, and D1246) have also been identified and confirmed by *in vitro* studies. In Uganda, these mutations have been detected and shown to be on the increase among the *P. falciparum* population. This further shows the need to monitor emergence of resistance by the *P. falciparum* population to both drugs in the most commonly used ACT combinations.

1.3 Research questions

1. What are the different mutations in the K13 propeller gene of *P. falciparum* parasites isolated from symptomatic malaria patients prior to A-L treatment at Kasangati Health Centre IV, Wakiso district?

2. What is the proportion of patients who still have *P. falciparum* parasites in their blood on day seven (7) after taking a full dose of A-L antimalarial agent?

3. What is the association between mutations detected in the K13 propeller gene of *P. falciparum* parasites isolated from symptomatic malaria patients prior to taking A-L treatment and presence of *P. falciparum* parasites in the blood of

patients on day 7 after completing a full dose of A-L treatment at Kasangati HC IV, Wakiso district?

1.4 Study Objectives

1.4.1 General objective

To assess K13 propeller gene mutations among *P. falciparum* parasites isolated from symptomatic malaria patients on A-L treatment at Kasangati Health Centre IV, Wakiso district.

1.4.2 Specific objectives

1. To determine the prevalence of mutations in K13 propeller gene of *P. falciparum* parasites isolated from symptomatic malaria patients prior to taking A-L treatment at Kasangati Health Centre IV, Wakiso district.
2. To determine the proportion of patients with *P. falciparum* parasites on day seven (7) after taking a full dose of A-L antimalarial agent at Kasangati Health Center IV, Wakiso district.
3. To determine the association between mutations detected in K13 propeller gene of *P. falciparum* parasites isolated from symptomatic malaria patients prior to taking A-L treatment and presence of parasites in blood samples of patients on day 7 after completing a full dose of A-L treatment at Kasangati HC IV, Wakiso district.

1.5 Significance of the study

The findings from this study report on the status quo of *P. falciparum* resistance mutations to artemisinin agents in Uganda. The study provides knowledge on K13 gene mutations detected in the parasite population of patients getting treated at Kasangati HC IV. The findings from this study also provide baseline information from which decisions to manage or contain the threat of emergence of resistance to ACTs in Uganda can be derived. Based on the results of this, it demonstrates that there is need for regular monitoring of molecular resistance to artemisinin agents among *P. falciparum* parasites.

1.6 Justification of the study

In the recent past, there have been reports of resistance to artemisinin in Cambodia which is worrisome because resistance to chloroquine also emerged in the same area and within a decade had spread to most parts of the globe. Currently there are no effective alternative medicines to fall back on in case resistance to ACTs develops on a large scale. Therefore, there is a need to assess the possibility of development and spread of resistant parasites in Africa. Various factors that are rampant in Africa such as poor treatment adherence, use of substandard medicines among others could likely lead to development of resistance by the *P. falciparum* parasites. World Health Organization recommends that monitoring of artemisinin resistance be carried both phenotypically and genotypically on a regular basis in order to preserve and prolong the lifespan of this novel class of antimalarial agents. In Uganda ACTs are the recommended first line treatment for uncomplicated malaria. Therefore, there is a need for continuous monitoring of resistance to artemisinin-based agents especially in malaria endemic countries like Uganda which this study does.

1.7 Scope of the study

The study was cross-sectional and assessed the prevalence of *P. falciparum* K13 propeller gene mutations in parasites isolated from symptomatic malaria patients between June 2017 and August 2017. The study was conducted on patients receiving medical care at Kasangati Health Center IV in Wakiso district located in Central region of Uganda.

1.8 Conceptual framework

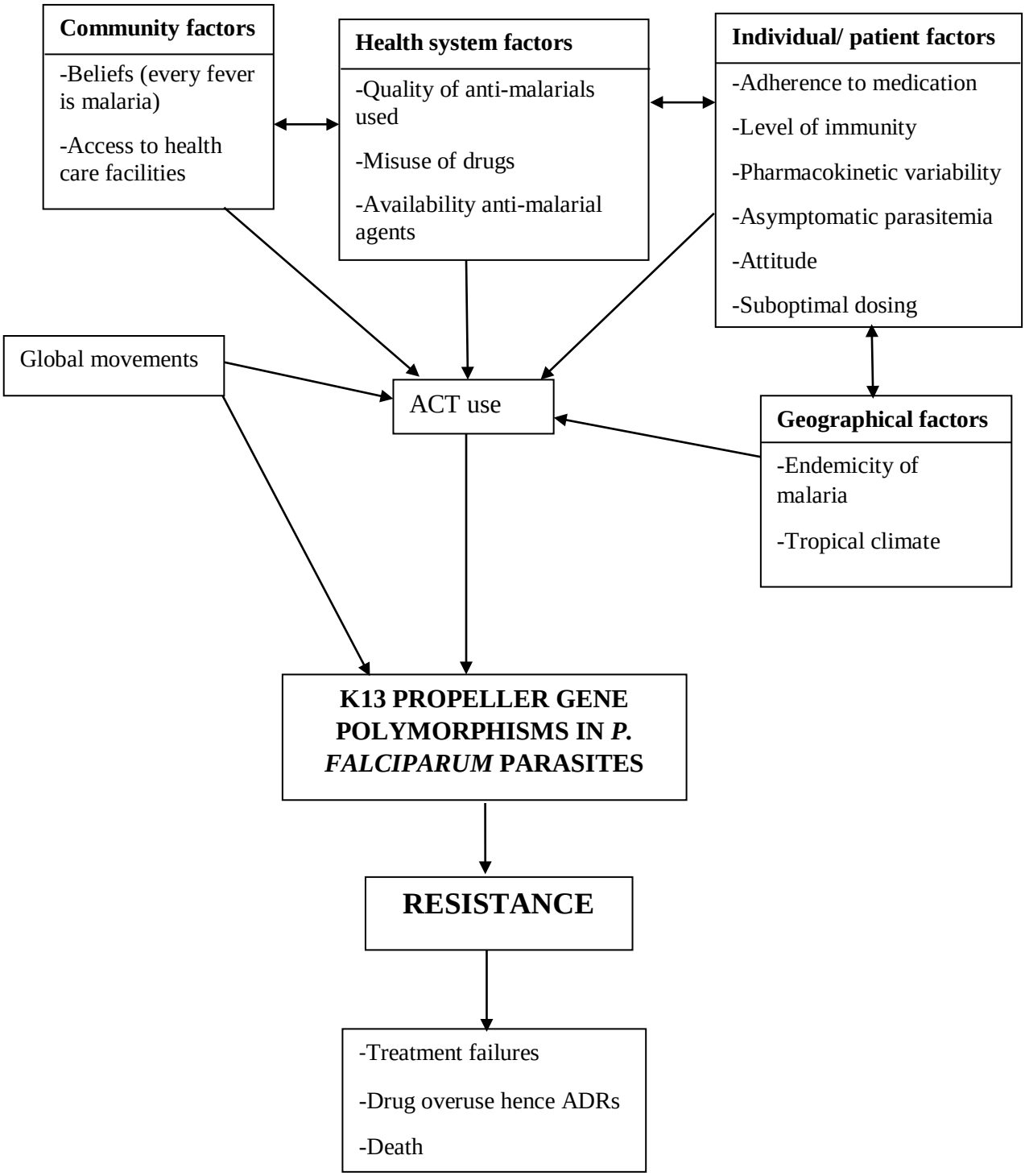


Figure 1: A conceptual frame work showing factors associated with artemisinin resistance.

The different categories of factors contribute to development of mutations in the *P. falciparum* K13 gene. Factors in these categories alter the way how individuals use these medications. Mutations in this gene are a good predictor of resistance to artemisinin and some mutations have been confirmed to confer resistance to artemisinin. Community factors affect how patients use ACTs for example the belief that every fever is malaria can compel one to first self-medicate in case of any fever before seeking proper medical care. This kind of practice lays the foundation for emergence of resistance. Increase in global movements has led to increased spread of resistant parasites to various areas thereby altering how medicines are used in these areas. This comes about when malaria asymptomatic individuals travel quite often. Geographical factors such as endemicity affects ACT use in that highly endemic areas tend to have more doses administered and this in turn leads to selection of strains that can survive this drug pressure. Individual factors such as suboptimal dosing, pharmacokinetic variability and adherence to medication can result in suboptimal drug levels within the body which provides a suitable environment for parasites to adapt to the drug. Health system factors can also drive the emergence for example in the event of regular drug stock outs, patients will not be able to receive full doses of the medication hence resulting in sub therapeutic drug levels. When resistance develops within a population, this can lead to treatment failures that can at times result in death if the condition is serious. Resistance can also lead to drug over use because in the event that treatment failure occurs, the medical practitioner may decide to re-administer the same medication. The medical practitioner may also decide to change the medication after failure of the first medication and if resistance had been appreciated in such a setting well in advance, a suitable regimen would have been administered in the first place.

CHAPTER TWO: LITERATURE REVIEW

2.1 Malaria and its impact

Malaria can be caused through an infection with either one of five species of *Plasmodium*, the life cycles of which are very similar. These species include: *Plasmodium vivax, P. falciparum, P. ovale, P. malariae* and *P. knowlesi* (Cox, 2010).

Malaria remains a disease of major concern causing significant health problems in many parts of the world, especially Africa. The World Health Organization estimated that there were 216 million cases of malaria in 2016 with about 445,000 deaths globally (World Health Organisation, 2018b). Total funding for malaria control and elimination reached an estimated 2.7 billion US dollars in 2016. Contributions from governments of endemic countries amounted to 800 million US dollars, representing 31% of funding (World Health Organisation, 2018b). *P. falciparum* is responsible for over 90% of the cases and almost all of the malaria deaths worldwide (Sinclair *et al*., 2009). Malaria still remains a major concern despite recent reports of a falling incidence of infection following deployment of ACTs and insecticide-treated bed nets in specific geographical settings (Muangnoicharoen, Johnson, Looareesuwan, Krudsood, & Ward, 2009). However, Sub-Saharan Africa still continues to carry a disproportionately high share of the global malaria burden. In 2016, the region was home to 90% of malaria cases and 91% of malaria deaths on a global scale (World Health Organisation, 2018b). Some 15 countries (Nigeria, Democratic Republic of the Congo, Burkina Faso, Mali, United Republic of Tanzania, Niger, Mozambique, Ghana, Uganda, Angola, Kenya, Cameroon, Guinea, Chad) mainly in sub-Saharan Africa except India have accounted for 80% of malaria cases and 78% deaths globally and since 2000, the decline in malaria incidence in these 15 countries (32%) has lagged behind that of other countries globally (53%) (World Health Organisation, 2018b).

Malaria's social and economic burden is a major obstacle to human development in most low and middle-income countries globally. In heavily affected countries, malaria alone accounts for as much as 40% of public health expenditure, 30% to 50% of hospital admissions, and up to 60% of outpatient visits (Sinclair *et al*., 2009). In areas with high transmission of malaria, children under five years of age are particularly susceptible to infection. Illness and deaths of more than

two thirds (70%) of all malaria deaths occur in this age group (World Health Organisation, 2018b).

P. falciparum malaria is of greatest importance in Africa because the vast majority of malaria cases (90%) and deaths (up to 99%) are due to *P. falciparum* malaria. Of the eighteen countries that account for 90 percent of *P. falciparum* infections in sub-Saharan Africa, Uganda ranks third (Uganda Ministry of Health, 2015; World Health Organisation, 2016). Malaria is the leading cause of morbidity and mortality accounting for 30-50% of outpatient visits, 15-20% of hospital admissions and 9-14% of inpatient deaths in the country (Ocan *et al.*, 2016; Uganda Ministry of Health, 2015). Up to 70% of outpatient cases and over 50% of inpatient admissions in the under-fives are malaria cases and malaria alone accounts for nearly half of all inpatient pediatric deaths (Kiwanuka, 2003; Ocan *et al.*, 2016; Uganda Ministry of Health, 2015). Malaria transmission has a stable, perennial pattern in 90 to 95 percent of Uganda though there are seasonal exacerbations and morbidity in the country (Kiwanuka, 2003; Uganda Ministry of Health, 2015).

Malaria may either be uncomplicated or severe depending on symptoms and also the parasite load. Uncomplicated malaria is the mild form of the disease which presents as a febrile illness with headache, tiredness, muscle pains, abdominal pains, rigors (severe shivering), and nausea and vomiting. (World Health Organisation, 2018b). If left untreated *P. falciparum* malaria can rapidly develop into severe malaria which presents with anemia (low hemoglobin in the blood), hypoglycemia (low blood sugar), renal failure (kidney failure), pulmonary edema (fluid in the lungs), convulsions (fitting), respiratory distress in relation to metabolic acidosis, coma, and eventually death can occur if not attended to (Sinclair *et al.*, 2009; World Health Organization, 2015a). Patients with *P. falciparum* density of more than 10,000 parasites /µl are considered to have severe malaria though some may present with signs and symptoms of uncomplicated malaria. Parasite density of more than 10% is considered as severe malaria everywhere even though they do not present with the accompanying signs and symptoms (World Health Organization, 2015a).

2.2 Malaria transmission

The intensity with which malaria is transmitted varies both naturally and as a consequence of human public health intervention (Hastings & Watkins, 2005). Malaria is transmitted through the

bites of infected female *Anopheles* mosquitoes. There are more than 400 different species of *Anopheles* mosquitoes; around 30 are malaria vectors of major importance. All of the important vector species bite between dusk and dawn (World Health Organisation, 2018b). Transmission is more intense in places where the mosquito lifespan is longer (so that the parasite has time to complete its development inside the mosquito). The long lifespan and strong propensity to bite humans of the African vector species is the main reason why nearly 90% of the world's malaria cases are in Africa (World Health Organisation, 2018b).

Transmission also depends on climatic conditions that may affect number and survival of the vectors, such as rainfall patterns, temperature and humidity. In many places, transmission is seasonal, with the peak during and just after the rainy season (Kiwanuka, 2003; World Health Organisation, 2018b). Malaria epidemics can occur when climate and other conditions suddenly favor transmission in areas where people have little or no immunity to malaria. They can also occur when people with low immunity move into areas with intense malaria transmission, for instance to find work, or as refugees (World Health Organisation, 2018b).

Human immunity is another important factor, especially among adults in areas of moderate or intense transmission conditions. Partial immunity is developed over years of exposure, and while it never provides complete protection, it does reduce the risk that malaria infection will cause severe disease. For this reason, most malaria deaths in Africa occur in young children, whereas in areas with less transmission and low immunity, all age groups are at risk (World Health Organisation, 2018b).

The intensity of transmission depends on factors related to the parasite, vector, human host, and environment (World Health Organisation, 2018b). The relationship between transmission intensity and the rate at which antimalarial drug resistance evolves usually affects the design of surveillance programs, and the likely impact of malaria control programs (Hastings & Watkins, 2005). However, the most important result is that transmission intensity does not directly affect the evolution of resistance (Hastings & Watkins, 2005). Therefore, it is the intensity of transmission after the agent has acquired resistance to an antimalarial drug that is of greatest concern (Hastings & Watkins, 2005). Due to the absence of an effective vaccine, malaria is controlled primarily by drugs which makes parasite resistance to artemisinin agents a vital concern for long term health. This particular concern affects a large number of the world’s

population, especially in Africa, where most of the world's malarial infections and deaths occur (Hastings & Watkins, 2005).

2.3 Evolution of antimalarial drug resistance

Antimicrobial resistance can be defined as infectious microbes that have acquired the ability to survive exposures to clinically relevant concentrations of antimicrobial drugs that would kill otherwise sensitive organisms of the same strain (Mack, Relman, & Choffnes, 2011). Several theoretical investigations have been made into the dynamics of how antimalarial resistance evolves, for example in various investigations by (Cross & Singer, 1991; Curtis & Otoo, 1986; Dye & Williams, 1997; Hastings, 1997; Hastings, Watkins, & White, 2002; Nicholas White, 1999) reviewed in Hastings & d'Alessandro, 2000. The results of these different investigations appeared to be remarkably similar (Hastings & Watkins, 2005). Several factors that contribute to the parasite developing resistance include: sexual recombination, intra-host dynamics, the level of drug use in the population, the proportion of malaria infections treated, and the number of parasites in a human host among others (Mackinnon & Hastings, 1998). In an influential study, Curtis & Otoo, (1986) showed that the prevalence of drug use was the most important factor in determining the rate of spread of resistance, and that resistance to two drugs would spread more slowly than resistance to one drug (Mackinnon & Hastings, 1998).

Drug resistance in malaria does not usually arise through a single mutational step, but more commonly arises at the end of a longer process during which parasites accumulate mutations and become ever more tolerant of the drug (Watkins & Mosobo, 1993). Increased drug tolerance allows these parasites to survive in humans containing residual levels of a drug taken sometime previously. Therefore, frequent use of antimalarial drugs in endemic areas means that many people harbor sub-therapeutic levels of the drug, which constitutes a potent selection pressure driving tolerant mutations in the parasite population (Watkins & Mosobo, 1993).

Malaria symptoms have been treated with drugs for hundreds and likely thousands of years, beginning with qinghausu in ancient China and quinine in the 17th century. During this early period, the only measure of drug efficacy was relief of patient symptoms after treatment (Sibley, 2014). The first widely used manufactured antimalarial drug was chloroquine. The drug was widely deployed after the second world war around 1944 - 1945 and has been used to treat

hundreds of millions of malaria patients (Sibley, 2014). The drug was cheap, non-toxic, and active against all strains of malaria parasites hence its wide spread use (Robert, Benoit-Vical, Dechy-Cabaret, & Meunier, 2001).

The success of chloroquine and its heavy use through the decades eventually led to chloroquine resistance in *P. falciparum* and *P. vivax*, the two parasite species responsible for most human malaria cases (Wellems & Plowe, 2001). Resistant *P. falciparum* parasites were detected in Colombia and at the Cambodia-Thailand border during the late 1950s. Resistant strains from these foci spread steadily in the 1960s and 1970s through South America, Southeast Asia, and India. Africa was spared until the late 1970s, when resistance was detected in Kenya and Tanzania; the sweep of resistant *P. falciparum* across that continent followed within a decade (Wellems & Plowe, 2001). Without a replacement drug having the low cost and reliability of chloroquine, morbidity and mortality resurged, notably among children in Africa (Wellems & Plowe, 2001).

Chloroquine resistance has been documented in almost all malaria endemic regions of the world today. There is also high-level resistance to SP throughout South East Asia and increasingly spreading in various parts of Africa. Mefloquine resistance is common in the border areas of Cambodia, Myanmar, and Thailand, but uncommon elsewhere (Sinclair *et al.*, 2009).

In the public health sector, antimalarial treatment efficacy is measured according to guidelines regularly updated by WHO. The measurement guidelines are however, expensive and time consuming whereby treated patients are followed for at least 28 days to ensure that all symptoms of the disease have resolved and parasites have been cleared from the patient's blood (Sibley, 2014).

In Uganda, the Ministry of Health set up several sentinel sites in 1997 with support from the East African Network on Monitoring Antimalarial Treatment and WHO to monitor the efficacy of anti-malarial agents. The sentinel sites represented all geographic, epidemiological, and ecological strata of malaria in Uganda (Nabyonga-Orem, Ssengooba, Macq, & Criel, 2014). Evidence from these sentinel sites showed that resistance to chloroquine exceeded the WHO recommended threshold beyond which a policy change is recommended. Thus, several countries, including Uganda, embarked on changing their malaria treatment policies (Bukirwa *et al.*, 2006;

Nabyonga-Orem *et al*., 2014). In Uganda the treatment policy changed from chloroquine to a combination of chloroquine + SP in June 2000 and in 2002 replaced chloroquine as the first-line recommended therapy for uncomplicated malaria (Bukirwa *et al*., 2006; Nabyonga-Orem *et al*., 2014). Due to increasing resistance to chloroquine + SP, the treatment policy was again changed to ACTs, specifically AL, as the first-line treatment for uncomplicated malaria and artesunate-amodiaquine as an alternative. This process occurred over a period of 25 months, from March 2004 to April 2006 (Bukirwa *et al*., 2006; Nabyonga-Orem *et al*., 2014).

2.4 Artemisinin Combination Therapy

Historically, communities adopted monotherapy strategies for the treatment of malaria however, this was a very poor strategy. This is because looking at other infectious diseases, such as tuberculosis and HIV infection, combination chemotherapy is necessary for slowing resistance development (White, 1999). When used as monotherapy, the short half-life of the artemisinin derivatives (rapid elimination from the blood) means that patients must take the drug for at least seven days. Failure to complete the course, due to the rapid improvement in clinical symptoms, can lead to high levels of treatment failure even in the absence of drug resistance (Sinclair *et al*., 2009).

The rationale for combination chemotherapy in malaria is that if you have two or more drugs with independent mechanisms of action, then the probability of a parasite emerging that is resistant to both mechanisms at the same time is reduced significantly. This is so provided that parasites resistant to either component drug are rare in the population (White, 1999).

World Health Organization championed the use of combination chemotherapy for the treatment of malaria paving way for the recommendation of ACT use in 2003 (Adhin *et al*., 2012). These combinations should include an artemisinin-based drug, such as artesunate, artemether, dihydroartemisinin, or artemisinin. This recommendation was based on the fact that resistance to the other anti-malarials had risen far beyond the acceptable threshold (Muangnoicharoen *et al*., 2009).

There are a number of ACTs currently available for use including: Artemether Lumefantrine (AL), dihydroartemisinin (DHA)-piperaquine, Artesunate-Mefloquine (AM), Artesunate-Sulfadoxine-pyrimethamine, Artesunate-Amodiaquine. ACTs are relatively safe with few serious

side effects (Taylor & Juliano, 2014). Minor side effects are more common but can be difficult to distinguish from the symptoms of malaria. More so artemisinin derivatives have been shown to produce faster relief of clinical symptoms and faster clearance of parasites from the blood than other antimalarial drugs. For uncomplicated *falciparum* malaria, treatment typically entails a short course of an ACT. In 2013, ACTs were administered in more than 330 million courses globally (Taylor & Juliano, 2014). There are several dosing routes for artemisinin and its derivatives, including IV, IM, oral and rectal. Artesunate is suitable for administration through all routes while artemether can be used by IM, oral or rectal route. Oral bioavailability is 30% due to high first-pass metabolism (Medhi, Patyar, Rao, Ds, & Prakash, 2009). Artemether reaches its peak in 2–6 hours. There is modest plasma protein binding ranging from 43 to 81.5%. Artemether is extensively metabolized and converted to dihydroartemisinin which has a plasma half-life of 1–2 hours. This derivative accounts for much of antimalarial activity (Medhi *et al*., 2009). Artemisinin is characterized by a rapid onset of schizonticidal action and a short half-life. Parasite clearance is, however, often incomplete when it is employed as a single agent unless high dosages are used over several days. Such a regimen may reduce patient compliance and increase the danger of toxicity (Medhi *et al*., 2009).

Lumefantrine has variable bioavailability, largely due to fat-dependent absorption, with high plasma protein binding (mainly to high-density lipoproteins). It is extensively metabolized in the liver, primarily by the CYP3A4 enzymes (PK Group, 2015). Lumefantrine concentration on day 7 has been shown to be the most important single concentration measure and its association with treatment response. The 'therapeutic' day 7 lumefantrine concentrations published to date range from 170 ng/ml to 500 ng/ml (PK Group, 2015).

Despite the advantages that the ACTs possess, there are also some downsides such as when AL is compared to chloroquine, AL has a higher pill burden which potentially results in poor adherence and so does the frequency and duration of therapy result in poor adherence (Mace *et al*., 2011; Shorinwa & Ebong, 2012). More so the cost of AL is quite high as compared to chloroquine and this contributes to inadequate stock at health centers thereby limiting access (Sipilanyambe *et al*., 2008). Meanwhile other individuals complain that the large size and the unpleasant taste of the medication affect their adherence to treatment (Shorinwa & Ebong, 2012).

2.4.1 Mechanism of action of Artemisinin

Artemisinin and its derivatives are selectively toxic to malaria parasites. One reason for this selectivity is the enhanced uptake of the drug by the parasite. *P. falciparum* infected erythrocytes concentrate dihydroartemisinin and artemisinin to more than 100-fold higher concentration than do uninfected erythrocytes (Robert, Benoit-Vical, & Dechy-Cabaret, 2001). An artemisinin derivative lacking the endoperoxide bridge (deoxy-artemisinin) is devoid of antimalarial activity. Hence the possible reactivity of this peroxide function within the parasite is the key factor of the pharmacological activity of these molecules (Robert, Benoit-Vical, & Dechy-Cabaret, 2001).

Artemisinins require to be active once they reach the site of action. They can be bio-activated in one of two models to generate carbon-centered free radicals or reactive oxygen species (Cui & Su, 2009). Reductive scission model whereby low valent transition ions (ferrous heme or non-heme exogenous Fe^{2+}) bind to artemisinin. And after subsequent electron transfer induce reductive scission of the peroxide bridge to produce oxygen centered radicals which rearrange to give carbon centered radicals (Cui & Su, 2009; O'neill, Barton, & Ward, 2010). The other model, Open peroxide model suggests that ring opening is driven by protonation of the peroxide or by complexation by Fe^{2+}. In addition, it has also been suggested that non-peroxidic oxygen plays a role in facilitating ring opening of the peroxide to generate the open hydro-peroxide. The oxygen atom provides stabilization of the positive charge and, according to transition state theory, lowers the energy required for ring opening (O'neill *et al.*, 2010).

Once the drug has been activated, there are several proposed parasite molecular targets which include: Heme, whereby *in vitro* alkylation of heme by artemisinin and in the presence of red cell membranes was shown to cause oxidation of protein thiols. Besides heme, studies have also shown radiolabeled artemisinin to react covalently with several parasitic proteins (O'neill *et al.*, 2010). Another target of artemisinin is inhibition of *P. falciparum* ATPase enzyme which is an orthologue to mammalian Sarco/Endoplasmic Reticulum Membrane Calcium ATP-ase (SERCA). The role of this enzyme is to reduce cytosolic free calcium concentrations by actively concentrating Ca^{2+} into membrane bound stores, an activity critical to cellular survival (O'neill *et al.*, 2010; Wang *et al.*, 2015). Another target of artemisinins are the parasite membranes and artemisinin has been shown to accumulate within neutral lipids and cause parasite membrane damage. Components of the electron transport chain have also been shown to be susceptible to

artemisinin (O'neill *et al*., 2010). Recently, a reversible artemisinin target, *P. falciparum* phosphatidylinositol-3-kinase (*Pf*PI3K), has also been identified as a target whereby the enzyme is inhibited (Wang *et al*., 2015).

2.4.2 Artemisinin resistance

Parasite resistance, especially of *P. falciparum*, has been recorded to every utilized anti-malarial drug (Adhin *et al*., 2012). This calls for a diligent monitoring of emerging artemisinin resistance in any malaria endemic area especially in light of the large parasite populations increasingly being exposed to artemisinin agents worldwide. The emergence of artemisinin resistance in parts of the Greater Mekong Sub-region such as Western Cambodia and Thailand therefore, means that this resistance could spread to other areas and hence the need for more routine monitoring (Adhin *et al*., 2012).

The term "artemisinin resistance" in *P. falciparum* has not been precisely defined. The clinical interpretation is a relatively slow parasite clearance rate in patients receiving artemisinin or ACT (Dondorp *et al*., 2009). In 2015, WHO divided the definition of artemisinin resistance into: 1) Suspected artemisinin resistance defined as a high prevalence of the delayed parasite clearance phenotype, or high prevalence of K13 mutants; and 2) Confirmed artemisinin resistance defined as a combination of delayed parasite clearance and K13 resistance-associated mutations in a single patient. Confounding factors in these definitions include the effect of partner drugs, immunity, insufficient levels of drug in the blood and non-validated K13 mutations (World Health Organization, 2015b)

In vitro studies have sought to find a suitable molecular marker to identify artemisinin resistance in *P. falciparum* parasites. Mutations and variable expression of several genes such as *Pf*MDR and *Pf*ATPase6 have been suggested but there is no concrete evidence that these mutations confer resistance to artemisinin (Mohon *et al*., 2014). A number of mutant alleles in the K13 propeller gene PF3D7_1343700 (PF13_0238), however, have been linked to *in vitro* artemisinin resistance and *in vivo* slow parasite clearance rate. They have therefore, been proposed as markers for artemisinin resistance (Dondorp *et al*., 2009). A very low rate of transmission can result in inadequate immunity to clear the parasites that might have survived after treatment with

an artemisinin agent, thereby increasing the selection pressure for resistant clones. Hence monitoring of possible resistant parasite clones is of paramount importance (Mohon *et al.*, 2014).

With one of the proposed mechanisms of artemisinin action being that it is a potent inhibitor of *P. falciparum* phosphatidylinositol-3-kinase (*Pf*PI3K), the mutation C580Y in *P. falciparum* kelch13 (*Pf*kelch13) gene is responsible for reduction in poly-ubiquitination of *Pf*PI3K and its binding to *Pf*Kelch13 (Lin & Zaw, 2015). This in turn results in a normal level of *Pf*PI3K with consequent normal metabolism of the parasite. This shows that certain mutations in the kelch 13 gene are able to give rise to the loss of the effect of artemisinin agents on the parasites (Lin & Zaw, 2015).

Various mutations (synonymous and non-synonymous) in the K13 gene of *P. falciparum* have been observed around the globe. In South-East Asia where resistance is reported to have emerged, distinct alleles originating from multiple independent events of emergence have been observed. In the eastern Greater Mekong Sub region (GMS) comprising Cambodia, Lao People's Democratic Republic and Viet Nam, mutations C580Y, R539T, Y493H and I543T that have been confirmed to confer resistance appear to be frequent (World Health Organization, 2015b). In the western GMS comprising China, Myanmar and Thailand mutations F446L, N458Y, P574L and R561H some of which have been associated with delayed parasite clearance are more common (World Health Organization, 2015b). In Africa, non-synonymous mutations are rare but highly diverse. Non-synonymous K13 mutations have been reported in Cameroon, Central African Republic, Chad, Comoros, Democratic Republic of the Congo, Gabon, Gambia, Kenya, Madagascar, Malawi, Mali, Rwanda, Togo, Uganda and Zambia. The most frequent allele observed in Africa is A578S however, none of the mutations conferring resistance to artemisinin have been observed in Africa (World Health Organization, 2015b).

In Cambodia where resistance emerged, studies determined that the parasite clearance half-life in patients with wild type parasites was significantly shorter (median 3.30 h, interquartile range 2.59–3.95) than those with C580Y (7.19 h, 6.47–8.31, P,1026,), R539T (6.64 h, 6.00–6.72, P,1024) or Y493H (6.28 h,5.37–7.14, P,1026) mutant parasites (Ariey *et al.*, 2014). More so, the parasite clearance half-life in patients carrying the C580Y parasites was significantly longer than those with Y493H parasites (P, 50.007, Mann–Whitney U test). This data indicates that C580Y,

R539T and Y493H select for slow clearing parasites in malaria patients treated with artemisinin agents (Ariey *et al*., 2014).

In Uganda, one study that isolated the K13 propeller domain in *P. falciparum* from 133 episodes in children diagnosed between 2010-12 in Tororo district observed five nonsynonymous mutations (Conrad *et al*., 2014). The observed mutations were in K13-propeller blade 5, and one in each of the blades 1, 3 and 4. The mutations were A617T, L619S, I465T, Y558H, and A578S respectively (Conrad *et al*., 2014). However, clearance of parasites after treatment with AL was generally prompt and parasitemia was present in only 13/175 patients more than two days after the initiation of treatment (Conrad *et al*., 2014). These findings indicated the absence of mutations associated with delayed parasite clearance in this parasite population in Uganda.

In another study in northern Uganda, out of sixty parasite DNA samples that successfully amplified, Single Nucleotide Polymorphisms (SNPs) were found in the K13-propeller gene of four samples at the positions; 509, 522, and 533 (Ocan *et al*., 2016). Of the four samples that had mutations, two had the same synonymous mutations (GAG to GAA) and two nonsynonymous mutations S522R and G533C. The mutation at codon 533 had previously been reported in *P. falciparum* parasites in Cambodia and was also found in this study however, it was not associated with delayed parasite clearance (Ocan *et al*., 2016).

Not all non-synonymous mutations in the K13 propeller gene so far reported indicate artemisinin resistance. Such mutants can represent “passer-by” genotypes; that is, they do not lead to selection of the mutant K13 genotype (World Health Organization, 2015b). Table 1 shows mutations that have been validated as resistance mutations and those associated with resistance. In addition, the position of the mutation affects the clearance phenotype. Validation of a K13 mutation as a resistance marker will require correlation with slow clearance in clinical studies, reduced drug sensitivity in *ex vivo* or *in vitro* assays (World Health Organization, 2015b).

Table 1: Associated and validated *P. falciparum* K13 resistance mutations to artemisinin (World Health Organization, 2015b)

K13 mutation	Classification
441L	Associated
446I	Associated
449A	Associated
458Y	Associated
493H	Confirmed
539T	Confirmed
543T	Confirmed
553L	Associated
561H	Associated
568G	Associated
574L	Associated
580Y	Confirmed
675V	Associated

Studies have also shown that cysteine protease falcipain-2 (FP2; *PF*3D7_1115700) is a principal *P. falciparum* hemoglobinase. Inhibition of this protease or knockout of the gene seems to block hemoglobin hydrolysis in trophozoites and hence leading to decreased artemisinin activity, as hemoglobin is required for a potent antimalarial effect (Conrad *et al.*, 2014; Sijwali & Rosenthal, 2004). Interestingly, parasites selected *in vitro* for artemisinin resistance had a nonsense mutation at codon 69 of the FP2 gene, suggesting that parasites partially blocked hemoglobin processing to limit toxicity from artemisinin (Conrad *et al*., 2014).

2.5 *Plasmodium falciparum* K13 propeller gene

The complete *P. falciparum* genome comprises 23 million base pairs, 80% A-T rich genome consisting of around 5000 genes on 14 chromosomes. The kelch13 propeller gene is found on chromosome 13 (Gardner *et al*., 2002). The K13 propeller gene has been predicted to consist of three domains about 225 amino acids in length: i) *Plasmodium* specific and well conserved N-terminal domain; ii) a BTB/POZ domain; and, iii) a six-blade C-terminal propeller domain formed of canonical kelch motifs. Kelch motif containing proteins are evolutionarily conserved across different species and grouped into KLHL type proteins (Mohon *et al*., 2014).

The *homo Sapien* contains 42 of these KLHL-type proteins and KLHL19, also known as KEAP1 protein, and there is maximum homology with *Plasmodium* K13 protein (Baird & Dinkova-Kostova, 2011). The human KEAP1 protein is a negative regulator of the inducible nuclear erythroid 2-related factor 2 (Nrf2)-dependent cyto-protective response, sequestering Nrf2 in the cytoplasm under steady state. Nrf2 is a bZip transcription factor and a member of the Cap 'n' Collar family of regulatory proteins (Baird & Dinkova-Kostova, 2011). Nrf2 is activated by changes in the redox state of the cell and functions to restore homeostasis by upregulating antioxidant, xenobiotic metabolizing, and other cyto-protective enzymes through the control of gene expression of a family of cyto-protective proteins (Baird & Dinkova-Kostova, 2011). The transcription factor Nrf2 binds to the antioxidant response element (ARE) present in promoters of genes involved in phase II detoxification and oxidative stress responses (Mohon *et al*., 2014).

Nrf2 forms a heterodimer to activate Maf transcription factor protein that binds to the ARE and activates transcription through the Maf recognition element (MARE) (Kansanen, Kuosmanen, Leinonen, & Levonen, 2013). The Keap1-Nrf2 pathway allows adaptation and survival following various conditions of chemical and physical stress by controlling gene expression of a large and diverse family of cyto-protective proteins. These proteins orchestrate a network of reactions that, in almost all cases, collectively result in protection against electrophiles and oxidants, and in enhanced cell survival (Baird & Dinkova-Kostova, 2011). Therefore, like the KEAP1 protein in humans, it is assumed that the K13 propeller performs a similar function in the *P. falciparum.* Kelch13 mutations therefore result in a prolonged ring stage survival with an enhanced stress response. The level of drug activation is also low at the ring stage (Wang *et al*., 2015). Parasites with Kelch13 mutations are able to overcome protein damage due to drug modifications by

activating stress responses hence allowing the parasite to survive artemisinin induced stress (Wang *et al*., 2015). However, no orthologue of Nrf2 has yet been identified in *Plasmodium* parasite genome (Mohon *et al*., 2014).

2.6 Lumefantrine Resistance

Lumefantrine resistance has been studied *in vitro* and has been associated with mutations in the *P. falciparum* multidrug resistance gene 1 (*pf*mdr1). The development of resistance against long half-life partner drugs postulated to occur through the posttreatment selection of less sensitive parasites, as reinfections are exposed to subtherapeutic blood drug levels of these slowly eliminated drugs (Malmberg *et al*., 2012). In a study by Malmberg *et al.,* 2012, it was observed that parasites carrying *pf*mdr1 N86, 184F, and D1246 pure alleles were able to survive at significantly higher median estimated lumefantrine blood concentrations. The highest estimated lumefantrine concentration that re-infecting parasites carrying N86 versus those carrying 86Y could withstand, differed by a factor of 35 (1184.3 nM and 34.3 nM, respectively). The influence of Y184F and D1246Y SNPs on drug susceptibility was less clear-cut, with the "sensitive" Y184 and 1246Y parasites able to withstand the highest drug levels (1184.3 nM and 1081.5 nM, respectively) (Malmberg *et al*., 2012). An increase in these mutations among *P. falciparum* parasite populations in Tanzania (Sisowath *et al*., 2005) and Uganda (Dokomajilar, Nsobya, Greenhouse, Rosenthal, & Dorsey, 2006) have been previously reported especially after AL treatment. Another study reported that these mutations are increasing in the *P. falciparum* parasite population in Uganda consistent with increasing use of AL (Taylor *et al*., 2017).

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study design

This was a nested cross-sectional study with a laboratory component that aimed at identifying mutations in the kelch 13 (K13) propeller gene of *P. falciparum* parasites isolated from symptomatic patients attending care at Kasangati Health Centre IV outpatient department and their possible effect on treatment outcome. The main study is titled "**PHARMACOVIGILANCE OF ANTIMICROBIAL AGENTS IN UGANDA: EMPHASIS ON ARTEMISININ COMBINATION THERAPIES FOR MALARIA TREATMENT."** The aims of the main study sought to perform prescription event monitoring and investigated local perceptions of health workers on the therapeutic failure rates and drug related predictors for commonly used ACTs in Uganda. The main study also intended to map and predict ACT resistance trends as well as evaluating the safety profiles and efficacy of 5-day and 7-day ACT regimens against the standard 3-day regimen in treatment of uncomplicated malaria. Study coordination was done at department of pharmacology and therapeutic, Makerere University while study participant recruitment was done at six randomly selected health facilities.

The selection criterion of the main study included adult patients 18- 45 years suffering from non-complicated malaria and had the ability to give informed consent. Meanwhile the excluded patients comprised anemic patients (Hb<7g/Dl), Co-morbidities known to influence immunity such as HIV, TB, Co-infection with Hepatitis, Severe liver disease (ALT $\geq$ 2.5 of the upper normal limit), Patients with severe malaria, liver dysfunction, Patients receiving strong inducers of CYP3A4 such as Rifampin, phenytoin and the inability to give informed consent for any reason.

This study was a laboratory-based study from which both qualitative and quantitative data was generated.

3.2 Study site and setting

This study was conducted in Central Uganda in the district of Wakiso at Kasangati Health Centre IV. Kasangati Health Center IV is located approximately 16.5 kilometers (10.3 miles)

north of Kampala city center on the Kampala-Gayaza Road and it is a public health facility. The incidence of malaria in this region was reported to be at around 3056 cases in 2016 however, there was a low rate of reporting which fell below 80% of all cases in the district (Uganda Ministry of Health, 2016).

Laboratory work that involved DNA extraction, DNA amplification and electrophoresis was performed at the Molecular Diagnostic Laboratory (MDL) located at the department of Microbiology, College of Health Sciences (MAKCHS), Makerere University, Kampala, Uganda. Sequencing of the nested PCR products was done at ACGT, Inc located at 35 Waltz Drive Wheeling, IL 60090 Germantown, MD Laboratory: 12321 Middlebrook Road, Suite 125 Germantown, MD 20874 (USA).

3.3 Study population and sample size

3.3.1 Study population

The study was done among adult (≥18 years) malaria symptomatic patients attending medical care at Kasangati Health Center IV, Wakiso district. This population was chosen because of the increase in malaria cases between 2015 and 2016 (Uganda Ministry of Health, 2016). Adult patients were chosen because they are more likely to self-medicate prior to seeking medical care and this results in them having residual drug concentrations in blood which drives emergence of resistance (Jovel, Kofoed, Rombo, Rodrigues, & Ursing, 2014).

3.3.2 Sample size determination

This study selected a total of one hundred (100) samples collected from patients that tested positive for *P. falciparum* malaria at Kasangati Health Center IV. A total of 1466 patients were screened for *P. falciparum* malaria at Kasangati Health Center IV in Wakiso district between June and August. The population size in the study area approximates 142, 361 inhabitants (Uganda Bureau of Statistics, 2016).

3.3.3 Sampling Criteria

The study population consisted of adult (≥18 years) malaria symptomatic patients attending medical care at Kasangati Health Center IV, Wakiso district. 1466 adult patients were referred to the laboratory for blood tests by the attending clinicians during the study period.

All these patients were screened for malaria using microscopy and those found to be positive for uncomplicated *P. falciparum* malaria were purposively sampled. Those who agreed to join the study were then inducted into the study. Uncomplicated malaria was determined based on the parasite load. Patients with parasite count of ≤ 230 parasites per 200 observed WBCs which equated to ≤ 9,200 parasites per microliter of blood were eligible for the study. Details of the study were explained to the patients and upon obtaining written informed consent, the patients were recruited into the study one after the other. Patients who joined the study were asked to return to the health facility after seven days in order to assess their treatment success. Treatment plan of the study participants was offered by the clinicians at the health center. Patients that did not meet the study criterion and patients with other illnesses were also managed accordingly by the clinicians.

3.3.4 Selection criteria

3.3.4.1 Inclusion criteria

- Adult patients above 18 years (including 4 pregnant women).
- Patients with uncomplicated malaria (parasite load of ≤ 230 parasites per 200 observed WBCs).

3.3.4.2 Exclusion criteria

- Patients unable to give consent to the study.
- Co-morbidities known to influence immunity such as HIV and TB.

3.3.5 Study variables

3.3.5.1 Dependent variables

- K13 propeller gene mutations
- Parasite clearance by day 7 after taking a full dose of artemether-lumefantrine treatment

3.3.5.2 Independent variables

- Patient gender
- Geographical region in the country
- Age of the study participants

3.4 Materials and reagents

3.4.1 Materials used

Blood sample collection and laboratory tests: Filter papers (Whatman™ 903 Protein Saver Cards, GE Healthcare Ltd, UK), Microscope, Rack.

DNA extraction: Single hole punching machine, paper towels, Heating Block.

PCR analysis: Micropipettes, pipette tips, Eppendorf tubes, PCR tubes, disposable gloves, water bath, centrifuge (Centrifuge 5430, USA), Thermocycler (Bio RAD T100, Singapore), Nanodrop spectrophotometer.

Gel electrophoresis: Parafilm, microwave, Transilluminator (BioDoc-it, imaging system, Upland CA, USA), Electrophoresis machine.

3.4.2 Reagents used

Blood sample collection and laboratory tests: 20X Giemsa stain (UNILAB, Kenya), Methanol, Distilled water.

DNA extraction: 10% Saponin, 1X Phosphate Buffered Saline (PBS), 20% Chelex, Sodium hypochlorite, distilled water, 70% ethanol

PCR analysis: 2X *Taq* master mix (Table 2), Primers (Eurofins, Germany) for primary and nested PCR (Table 3), Parasite DNA standard (3D7), Nuclease free water, Sample parasite DNA.

Gel electrophoresis: 1% Agarose (Fisher Scientific, USA), 1X TAE buffer (242g Tris, 57.1g acetic acid, 100ml of 0.5 EDTA), Ethidium Bromide (10mg/ml), 1kb ladder (500µg/ml), 6X

Loading dye (0.25 w/v bromophenol blue, 0.25 w/v xylene cyanol FF, 30% v/v glycerol in water).

3.5 Data collection

Data collection was carried out between the months of June and August 2017.

3.5.1 Laboratory diagnosis of malaria

3.5.1.1. Preparation of working Giemsa stain solution

A working Giemsa stain solution of 10% was prepared. 45mls of distilled water was poured into a clean beaker. The Giemsa stock solution was filtered into a clean container and using a clean pipette, 5mls of the Giemsa stock solution was transferred into the beaker containing the 45mls of distilled water. The working solution was prepared prior to staining of blood slides.

3.5.1.2. Screening for malaria parasites using thick blood smears

When preparing a slide, the third finger of the left hand was held and its tip wiped with spirit swab then allowed to dry. The finger was pricked with a disposable needle and blood allowed to ooze out onto the slide.

To detect the presence of parasites in patient blood, thick blood smears were used. The thick blood smears were prepared by spreading three drops (approximately half a milliliter) using a spreader of the patient's capillary blood on the microscope slides. The thick smears were dried under the heat of a 75-watt bulb and then the slides placed on a staining rack with the blood film facing up. Approximately 3mls of 10% Giemsa stain was gently poured onto the blood slides until it was fully covered with stain and left to stain for eight minutes. The stain was then gently flushed from the slides by adding drops of distilled water until all the stain had been washed away. The stained thick blood smears on slides were dried under the heat of the bulb and observed under the microscope at 100x oil immersion.

3.5.1.3. *Plasmodium* species identification and quantification using thin blood smears

Quantification and species determination of *Plasmodium* parasites was done using thin blood smears. Thin blood smears were prepared by dropping approximately a quarter of a milliliter

of the patient's capillary blood onto the slides and spreading using spreaders. The smears were then left to air dry in the laboratory. The thin smears were fixed by dipping the slides into a small container containing methanol. The slides were placed on a drying rack horizontally and the methanol-fixed thin smear allowed to dry completely in air. The slides were then placed on a staining rack with the blood film facing up and approximately 3mls of 10% Giemsa stain gently poured onto the blood film on the slide until it was fully covered with stain. The film was left to stain for eight minutes and thereafter the stain gently flushed from the slides by adding drops of water until all the stain had been washed away. The stained thin blood smears were then dried under the heat of the bulb and observed under the microscope at 100x oil immersion.

Trophozoites of *P. falciparum* are the smallest in size when compared to those of other *Plasmodium* species. The early trophozoite (ring) stage in *P. falciparum* infection appears as multiple rings in the red blood cell while the other species usually have one ring appearing in the red blood cell except for *P. vivax* that often has two rings (Appendix 2). Gametocytes of *P. falciparum* appear crescent shaped whereas gametocytes of the other *Plasmodium* species appear spherical.

In the quantification of parasites, *P. falciparum* parasites were counted per 200 observed white blood cells (WBCs). The parasite density was then calculated per microliter of blood using the formula below,

$$\text{Parasites / µl blood} = \frac{\text{Number of parasites counted } \mathbf{X} \text{ 8000 white blood cells/µl}}{\text{No. of white blood cells counted}}$$

Since a Complete Blood Count for every patient could not be carried out to determine the actual number of white blood cells, an average number of 8000 WBCs was used. This determination of parasite density in relation to White Blood Cells was done at the health center by the individual in charge of the laboratory. The laboratory assistant was a graduate in biomedical laboratory technology with over five years working experience in the laboratory.

3.5.2 Blood sample collection, transport and storage

Blood sample collection at the health center was done between 9 AM and 4 PM from Monday to Saturday during the study period. Patients that had tested positive for *P. falciparum* malaria were informed of their disease status and after obtaining written informed consent, more capillary blood was taken. Four drops of blood were collected from each patient and spotted on filter paper Whatman™ 903 Protein Saver Cards (GE Healthcare Ltd, UK). The blood was then left to dry overnight on a rack in the laboratory. The filter papers with dried bloodspots were then placed in separate envelopes so as to avoid contamination. The filter papers were transported in the envelops and stored on shelves at room temperature in the molecular laboratory, department of Pharmacology & Therapeutics, Makerere University awaiting analysis.

3.5.3 DNA extraction

Parasite DNA was extracted using Chelex resin method from dried blood spots on filter papers as previously described by Plowe, 1995 (Plowe, Djimde, Bouare, Doumbo, & Wellems, 1995). Briefly, two 6mm dried blood filter paper discs were cut using a sterile single hole punching machine each from separate blood spots on the filter paper and placed into a 1.5ml Eppendorf tube. 1ml of 0.5% Saponin in 1X Phosphate Buffered Saline (PBS) was added to the punched bloodspot sections in each Eppendorf tube and each labeled tube inverted several times to thoroughly mix. The samples were incubated at 4^0C overnight.

The next morning samples were retrieved, thawed and centrifuged at 12000 rpm for 30 seconds and the supernatant discarded. 1ml of sterile 1XPBS without saponin was added to each sample and incubated at 4^0C for 30 minutes. The samples were again centrifuged at 12000 rpm for 30 seconds. The supernatant was removed and discarded. At this point the temperature of the heating block was also set to 95^0C and the holes in the heating block filled with distilled water. To each sample, 50µl of 20% chelex slurry was added followed by 100µl of nuclease free water. A negative control was prepared by adding the same amount of chelex and nuclease free water to a sterile 1.5ml Eppendorf tube. The samples were vigorously vortexed for 10 seconds and then centrifuged at maximum speed (13200rpm) for 15 seconds

to ensure that the sample was thoroughly mixed inside the slurry of the chelex. The samples were then incubated at 95^0C for 20 minutes.

The samples were vortexed for 15 seconds followed by centrifugation at maximum speed (13200 rpm) for 30 seconds so as to sediment all contents to the bottom of the Eppendorf tube. Then carefully, 50µl of supernatant was pipetted and transferred to a fresh sterile labelled microfuge tube and this was done for all the samples. This supernatant contained the DNA that was to be used in the PCR reactions and was stored at -20^0C until it was used. Pipetting chelex at this step would be inhibitory to PCR because chelex acts as a chelating agent for divalent ions. In PCR reactions, Mg^{2+} is important in the action of the polymerase enzyme and if chelex binds these ions, the PCR reaction may fail.

3.5.4 Amplification of DNA samples

Nested PCR, a method developed by Snounou *et al.*, 1993, that involves the use of two sets of primers and two rounds of PCR amplification (Primary and Secondary) was modified and used (Snounou *et al*., 1993). In this experiment, gelatin was not added to the reaction mix. Tween and glycerol were added to the reaction mix which were not present in the reaction developed by Snounou *et al*.

Table 2: Primer sequences used to amplify K13-propeller gene of *P. falciparum* isolates

Genes	Primers (Eurofins Genomics, Germany)
K13-propeller (p)	F: 5′-CGGAGTGACCAAATCTGGGA-3′ (Ariey *et al*., 2014)
	R: 5′-GGGAATCTGGTGGTAACAGC-3′ (Ariey *et al*., 2014)
K13-propeller (s)	F: 5′-GCCAAGCTGCCATTCATTTG-3′ (Ariey *et al*., 2014)
	R: 5′-GCCTTGTTGAAAGAAGCAGA-3′ (Ariey *et al*., 2014)

(P) Primary PCR reaction, (S) Secondary PCR reaction

F: Forward primer, R: Reverse primer

A master mix for the primary PCR reaction was prepared in a 1.5ml Eppendorf tube according to the volumes shown in table 3. The PCR tubes were labeled and 23µl of primary

master mix was added to each PCR tube. 2µl of sample DNA was added to each tube that contained primary master mix. A negative control to which no template was added and a positive control to which a known laboratory template (3D7) was added were included in the run. The tubes were sealed and the thermocycler was run according to the conditions listed in table 4.

Table 3: Master Mix calculation for Primary PCR for K13-propeller gene.

Reagent	Stock concentration	Final concentration	Volumes (µl)
2X *Taq* master mix	2X	1X	12.5
Forward Primer	10pmol	0.4pmol	1
Reverse Primer	10pmol	0.4pmol	1
Nuclease free water	-	-	8.5
***Template DNA**	150ng/µl (approx.)	12ng/µl	2
TOTAL VOLUME			**25**

*Template DNA was added separately

The working concentration of *Taq* master mix comprised 10mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$, 0.2mM dNTPs, 25 units/ml *Taq* DNA Polymerase, 5% Glycerol, 0.08% IGEPAL® CA-630, 0.05 % Tween®, pH 8.6 at 25°C.

Table 4: PCR thermo cycling conditions for primary PCR for K-13 Propeller gene

Step order	Cycle	Temperature (^{0}C)	Time (minutes)	No. of cycles
1	Initial Denaturation	94	5	1
2	Denaturation	94	0.45	35
3	Annealing	54	1	35
4	Extension	68	1.5	35
5	Final extension	68	5	1
6	Final hold	4^0C	∞	-

The master mix for the nested PCR was prepared in a 2ml Eppendorf tube according to the volumes shown in table 5. PCR tubes were labeled with corresponding labels to those used in primary PCR and 48µl of secondary master mix added to each tube. 2µl of primary PCR product was then added to each corresponding tube for the second PCR. Another negative control that contained the same amount of nested PCR master mix (48µl) but no template was added to the PCR run at this stage so as to ensure there was no contamination. The tubes were then sealed and the thermocycler was run according to the conditions listed in table 6.

Table 5: Master Mix calculation for nested PCR for K 13-propeller gene

Reagent	Stock concentration	Final concentration	Volumes (µl)
2X *Taq* master mix	2X	1X	25
Forward Primer	10pmol	0.4pmol	2
Reverse Primer	10pmol	0.4pmol	2
Nuclease free water	-	-	19
***Template DNA**	150ng/µl (approx.)	6ng/µl	2
TOTAL VOLUME			**50**

*Template DNA was added separately

Table 6: PCR thermo cycling conditions for nested PCR for K-13 propeller gene

Step order	Cycle	Temperature (^{0}C)	Time (minutes)	No. of cycles
1	Initial Denaturation	94	5	1
2	Denaturation	94	0.45	35
3	Annealing	54	1	35
4	Extension	68	1.5	35
5	Final extension	68	5	1
6	Final hold	4^0C	∞	-

The nested PCR products were separated using electrophoresis and viewed under UV light in a transilluminator to confirm whether DNA amplification was successful.

3.5.5 Gel Electrophoresis

To visualize the nested PCR products, electrophoresis was carried out on a 1% agarose gel (Fisher Scientific, USA). The gel was prepared by dissolving 1.5 grams of agarose in 150mL

of TAE buffer and microwaving the mixture for 3 minutes. The mixture was left to briefly cool and 5µl (10mg/ml) of ethidium bromide (Sigma Aldrich, USA) added. The mixture was swirled gently to uniformly mix the ethidium bromide. Thereafter, the mixture was poured into a casting tray in which a comb had been inserted. The mixture was then left to cool and solidify at room temperature for 30 minutes.

On solidification of the gel, inserted combs were taken out thereby creating wells. The gel was carefully transferred to the electrophoresis machine and immersed in approximately 1.5litres of 1X TAE buffer solution in the electrophoresis tank. 6µl of each nested PCR product was mixed with 2µl of loading dye (0.25 w/v bromophenol blue, 0.25 w/v xylene cyanol, 30% v/v glycerol in water) that had been dotted on a parafilm and then carefully loaded into the wells in the gel (each mixture deposited in a separate well). The wells at the extreme ends of the gel on either side were loaded with 5µl of DNA ladder. 1 kilobase pair and 100 base pair ladders were used. The loading dye helps in observation of the migrating DNA so as to prevent the DNA from running off the gel and also it enables the DNA to sink into the wells. A current of 110 volts was passed through the submerged gel for 35 minutes so as to allow DNA fragments of various sizes to separate. The gel was taken out and carefully placed in a trans-illuminator (BioDoc-it, imaging system, Upland CA, USA). This machine passed UV rays through the gel and DNA that had been stained with ethidium bromide was visible as bands on the gel. Nested PCR products that contained bands of the right size in reference to the DNA ladder were prepared for sequencing.

3.5.6 Sequencing of amplicons

DNA concentration in successfully amplified nested PCR products was measured using a nanodrop spectrophotometer. Thereafter, 50µl of the quantified DNA was aliquoted, packaged and shipped for sequencing. Sanger sequencing (BigDye terminator version 3.1) (Kan, Fredlake, Doherty, & Barron, 2004) was carried out at ACGT, Inc laboratories (35 Waltz Drive Wheeling, Illinois, USA).

3.6 Data management and Analysis

Electropherograms were analyzed for quality and FASTA formats of the sequences generated using SnapGene software (GSL Biotech LLC, Chicago, Ill., USA). The FASTA sequences

were imported into BioEdit software version 7.1.3.0 (Hall, 2011) for editing. Edited sequences were then exported to MEGA 6 software version 6.06 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013) and using the alignment tool Clustal W in MEGA 6, the sequences were aligned against a wild type sequence. The wild type K13 nucleotide sequence of *P. falciparum* was obtained from the NCBI database (GenBank) possessing the accession number, XM_001350122.1 against which the sequenced samples were compared during alignment. The aligned sequences in MEGA 6 were manually scanned for mutations. Mutations from the aligned sample sequences were analyzed as counts. Since there were no parasites observed on day seven, a statistical analysis could not be done to assess parasite presence on day seven and also correlation between mutations observed on day zero (0) and day parasite presence on day seven (7).

3.7 Quality Control

Practices that ensured high quality of data output were employed such as:

1. During laboratory diagnosis of malaria, an experienced laboratory technologist (over 5 years' experience) performed the microscopy.
2. Filter papers with dried blood spots were placed in different envelopes to prevent cross contamination between samples and they were transported and stored in their separate envelopes in the Molecular Diagnostic Laboratory.
3. In carrying out DNA amplification, negative controls were added to both PCR runs (primary and nested) to ensure that no foreign DNA was added to the samples. Positive laboratory controls which were characterized were also run alongside the samples for comparison.
4. During electrophoresis, positive and negative controls were also electrophoresed in order to confirm that there was no contamination and also that the nested PCR product was of appropriate size.
5. When preparing samples for sequencing, positive laboratory controls whose sequence was known and had been amplified alongside the samples was also shipped so as to make sure that observed mutations were not due to PCR errors.

6. Obtained sequences from sequencing were blasted into NCBI database to make sure that the sequenced amplicons were actually of the *P. falciparum* K-13 propeller gene.

3.8 Ethical Considerations

Ethical approval was sought from Makerere University College of Health Sciences, school of biomedical sciences, Research Ethics Committee (REC). The study was filed under the number SBS 376. Approval was also obtained from the Wakiso District Local Government and this was provided by the District Health Officer, Wakiso District. The reference number Ref: Med 218/06/2017 was awarded to the study. Consent from patients who qualified for the study was also sought.

CHAPTER FOUR: RESULTS

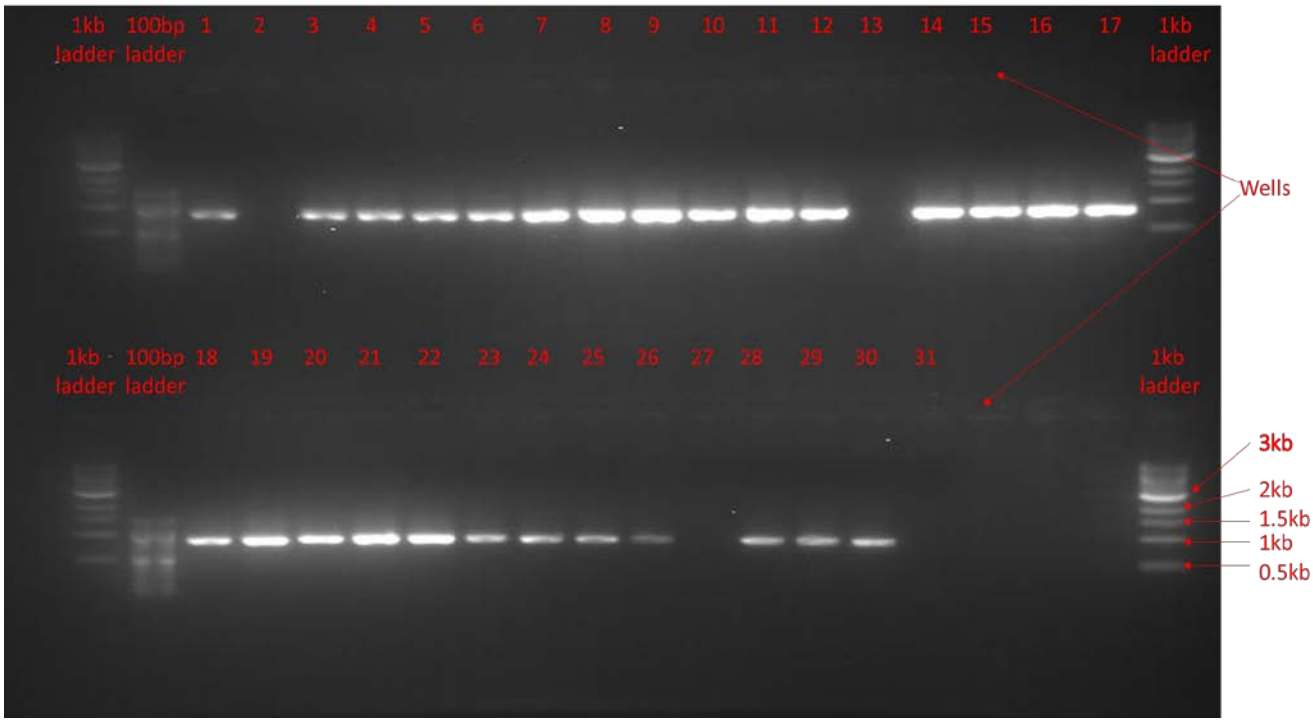
4.1 Study demographics

A total of 1466 adult out-patients were screened for *P. falciparum* malaria at Kasangati Health Center IV between June and August 2017. Majority, 75.6% (1109/1466) of the screened patients were females. Of the screened patients, 6.9% (102/1466) were positive for *P. falciparum* infection. All the patients with confirmed *P. falciparum* infection were recruited into the study except for two patients who did not consent. These two patients were not recruited into the study and continued with their treatment plan. Of the recruited patients, majority, 59% (59/100, P=0.84) were females. The average age (±SD) of the recruited study participants was 24.7 ± 9.3 years. There was no non-*P. falciparum* parasite malaria detected among the patients screened for *Plasmodium* infection.

4.2 Prevalence of K13 gene mutations (SNPs) in analyzed *P. falciparum* parasite DNA samples

Of the total 100 parasite DNA samples collected from patients on day zero, seventy (70) were successfully amplified (Figure 2). Amplicons from all the 70 samples were shipped for sequencing.

Figure 2: Electrophoresis gel image showing nested PCR products of K13 gene



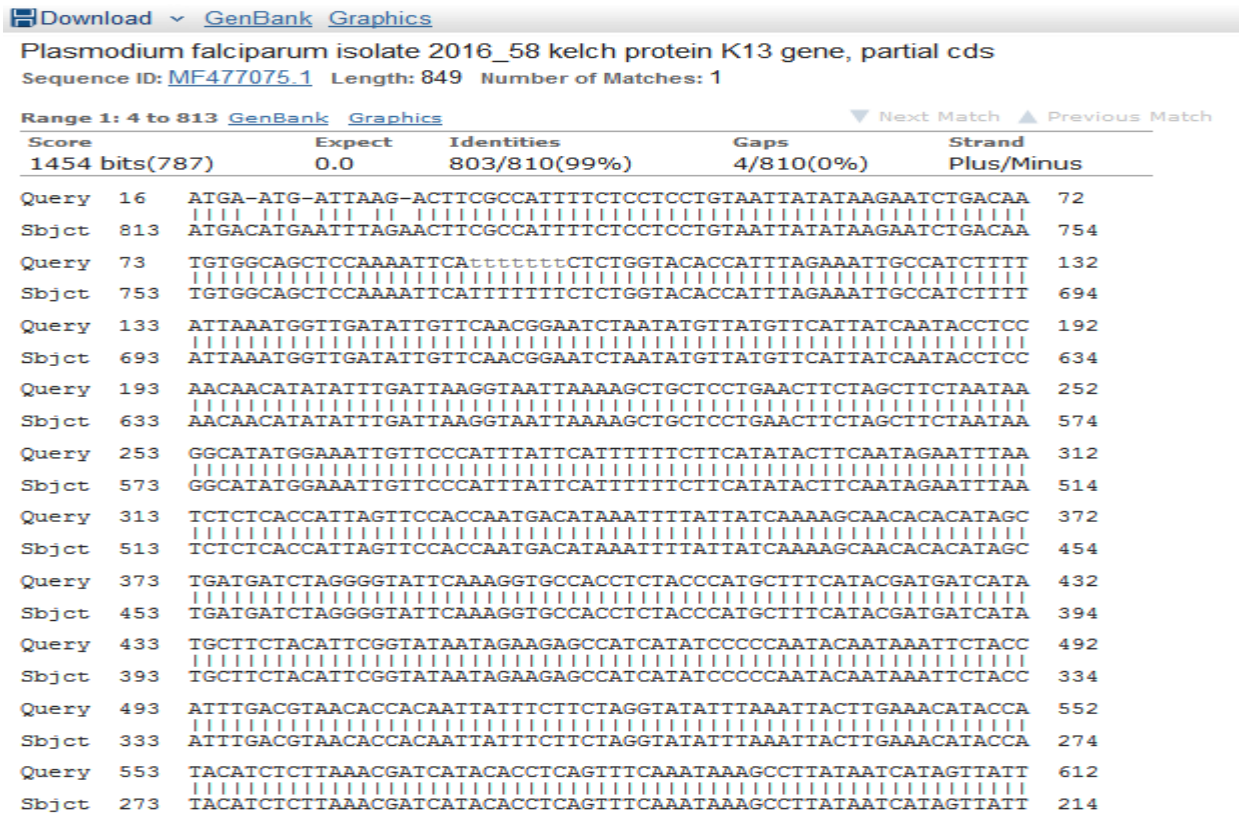
1-26: Represents the study samples, 27: Represents the negative PCR control for the first PCR reaction, 28-30: Represents the positive controls, 31: Represents the negative control for the second PCR reaction.

Figure 3: One of the Electropherograms showing the sequenced products

Of the seventy (70) amplicons, sixty-seven (67) were successfully sequenced and produced good electropherograms (Figure 4). Three (3) samples did not sequence appropriately or failed to sequence. Two (2) of the three samples failed to be sequenced while one (1) sample was successfully sequenced however, the resulting electropherogram was very noisy (many small undefined peaks on the electropherogram). The failure in sequencing could be attributed to DNA degradation during sample transportation.

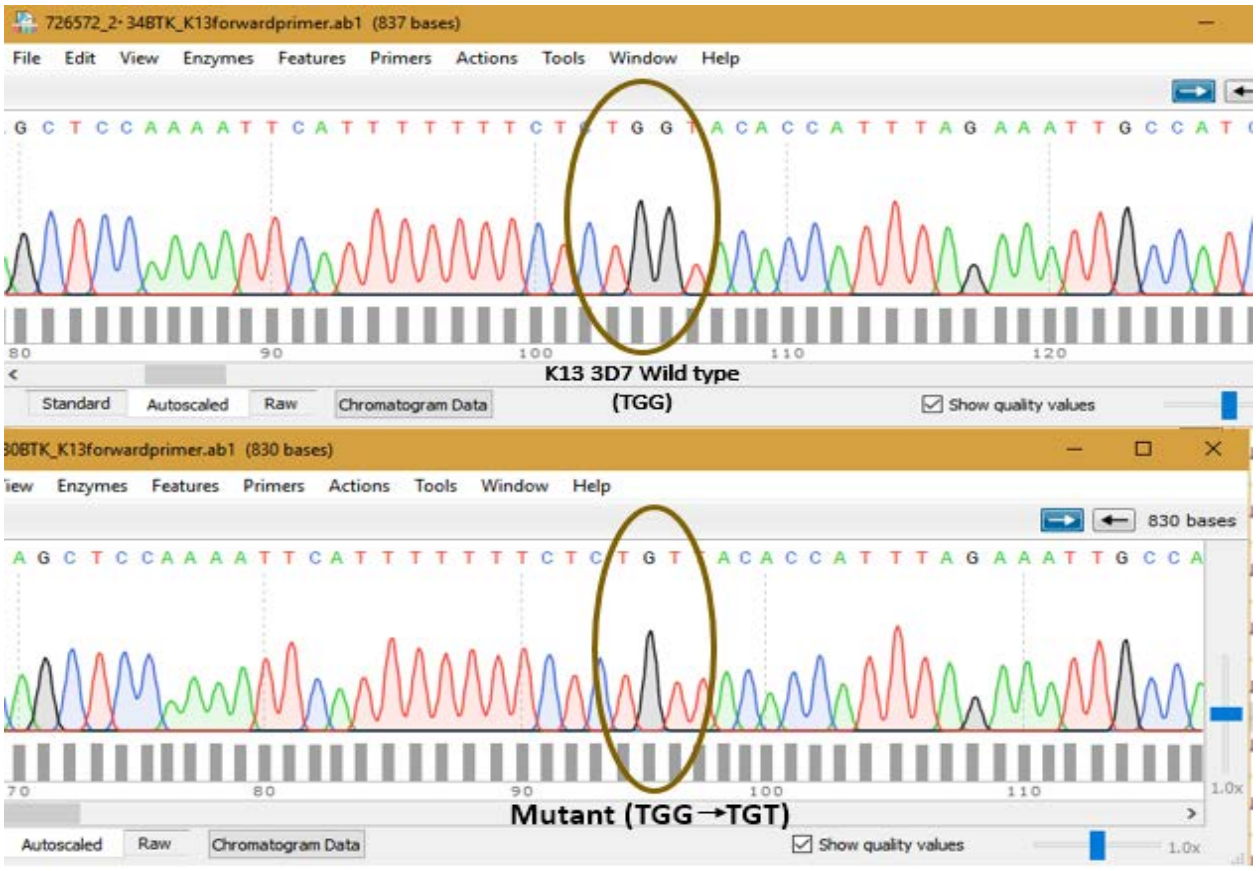
When the sequence products were blasted onto the NCBI database, the BLAST results showed a similarity of 99% over a query coverage of 787 nucleotides (figure 4).

Figure 4: NCBI BLAST output showing the similarity between sequenced product and the NCBI *P. falciparum* genome



A total of 67 samples were aligned against a reference *P. falciparum* K13 gene (3D7) obtained from the NCBI database (accession number: XM_001350122.1) and inspected for mutations. Single Nucleotide Polymorphisms (SNPs) were detected in two (2) samples. Both of the detected mutations were nonsynonymous (table 7).

Figure 5: Electropherogram showing a detected SNP in a sample



One mutation was detected at codon position 522 (nucleotide position 1563) while the other mutation was detected at codon position 667 (nucleotide position 2001) as shown in figure 6 and table 7.

Table 7: *P. falciparum* mutations observed in samples

Codon position	Nucleotide position	Reference codon	Mutant codon	Reference amino acid	Mutant amino acid	Nucleotide change	Prevalence of mutation % (n)
522	1563	AGT	TGT	Serine	Cysteine	A → T	1.5% (n=1)
667	2001	CCA	ACA	Proline	Threonine	C → A	1.5% (n=1)

4.3 Assessing *P. falciparum* parasite presence on day seven

Of the 100 recruited patients, 82% (82/100) returned to the health center on day seven (7) after completing treatment for follow-up re-assessment. Of the re-assessed patients, no *Plasmodium* parasites were detected in all the screened blood samples. Therefore, there was no association between the mutations detected in the *P. falciparum* parasites on day zero (0) and their persistence up to day seven (7) in the hosts.

CHAPTER FIVE: DISCUSSION

Resistance to artemisinin and its derivatives can be assessed either phenotypically or genotypically (Conrad *et al*., 2014; Dondorp *et al*., 2009; Ocan *et al*., 2016). Phenotypically, artemisinin resistance manifests as delayed parasite clearance and is also correlated with diminished action of pulses of artemisinins *in vitro* (Conrad *et al*., 2014; Straimer *et al*., 2015; Witkowski *et al*., 2013). Genetically, resistance to artemisinins can be determined through screening for the molecular biomarkers of resistance in particular genes that are known to be associated with delayed parasite clearance.

In the current study, nonsynonymous mutations were detected at codon positions 522 and 667 in the K13 propeller gene of *P. falciparum parasites.* Both mutations detected in the current study were uncommon. The mutation S522C detected in one of the samples caused the wildtype amino acid to change from serine to cysteine. In Uganda, three other studies reported mutations at the same codon position, one (a nonsynonymous mutation) in northern Uganda (Ocan *et al*., 2016) another (a synonymous mutation) in Eastern Uganda (Conrad *et al*., 2014) and also in a sub-Saharan study (Taylor *et al*., 2014). In both studies, it was reported that the detected mutations were not associated with delayed *in vivo* parasite clearance after treatment. Outside Uganda, this mutation was also detected in countries such as Togo (Dorkenoo *et al*., 2016) and the Democratic Republic of Congo (Mvumbi *et al*., 2017).

The other nonsynonymous mutation P667T that was detected in the current study was also uncommon and had been reported before. However, this mutation has not been reported in Uganda by any other study. The mutation caused the wildtype amino acid to change from proline to threonine. In Myanmar mutations at this codon position have been reported by two different studies (Imwong *et al*., 2017; Nyunt *et al*., 2017). The data from the current study showed that there are new mutations in the K13 propeller gene of *P. falciparum* population that are continuously being detected in Uganda. In other studies carried out in Uganda and also in the current study, mutations detected in the K13 propeller gene of *P. falciparum* parasites could have arisen due to the continuous selective pressure from artemisinin agents that were introduced into the country back in 2004 (Taylor *et al*., 2017).

There was a low frequency of mutations observed in this parasite population and this can be attributed to the fact that malaria-endemic regions of sub-Saharan Africa such as Wakiso district where the current study was done are less likely to be infected with clonal parasites. This is because of the high transmission intensity and the *Plasmodium* parasites are thus likely to lose potential resistance-conferring alleles to outcrossing (Daniels *et al*., 2013; Huang *et al*., 2015). In this case, outcrossing comes about when female *Anopheles* mosquitoes ingest haploid *P. falciparum* gametocytes during a blood meal from a human host. The gametocytes differentiate into gametes in the mosquito midgut, where they unite to form a diploid zygote, which in turn undergoes meiosis to restore haploidy prior to inoculation of the next human host (Daniels *et al*., 2013). Genetic outcrossing during the parasite's sexual stage occurs only when a mosquito bites a host infected simultaneously by multiple parasite strains and gametocytes from multiple genetically distinct strains that are circulating in the blood of a host (Daniels *et al*., 2013). Therefore, *P. falciparum* strains that may be resistant to artemisinin agents could be eliminated by the host's natural immunity when combined with treatment with an artemisinin agent.

The mutations C580Y, R539T, R543I and Y493H in the K13 propeller gene reported in Cambodia that were confirmed to be associated with parasite resistance to artemisinin (Ariey *et al*., 2014) were not detected in the current study. The observation in the current study is consistent with those in previous reports from Kenya (Isozumi *et al*., 2015), Angola (Escobar *et al*., 2015), Mozambique (Escobar *et al*., 2015), Senegal (Torrentino-Madamet *et al*., 2014), Uganda (Conrad *et al*., 2014; Ocan *et al*., 2016) as well as other areas of Sub-Saharan Africa (S. M. Taylor *et al*., 2014), Caribbean's Haiti (Carter *et al*., 2015), and South Asia' Bangladesh (Mohon *et al*., 2014), where the K13-propeller gene mutations associated with artemisinin resistance were not detected. These observed variations in existence of mutations in the K13 propeller gene of *P. falciparum* in various parts of the world is an indication of an emerging evolutionary adaptation to pressure by artemisinin agents among the parasite population.

It has been documented that changes in DNA sequences such as SNPs can alter protein structures which can in turn affect diverse protein properties, such as stability, catalytic activity or the ability to interact with other molecules depending on the site affected (Studer, Dessailly, & Orengo, 2013). Therefore, the mutations in the K13 gene of *P. falciparum* detected in this study could in one way or another result in an alteration in the way the parasites interact with

artemisinin agents. Therefore, whether detected mutations in the K13 propeller gene of *P. falciparum* parasites in different geographical regions outside Southeast Asia are associated with artemisinin resistance by delaying parasite clearance in malaria treatment by the artemisinin agents needs to be further investigated. This study was not able to establish whether the observed K13-propeller gene mutations in the *P. falciparum* parasite population in patients attending medical care at Kasangati Health center IV emerged spontaneously or spread to the study region from an outside Uganda. Resistance mutations can be greatly affected by transmission (Petersen, Eastman, & Lanzer, 2011). In areas of high transmission intensity, the host will have more polyclonal infections. This means a higher probability of transmitting gametocytes of multiple genetic backgrounds that will recombine in the mosquito (Escalante, Smith, & Kim, 2009). This is particularly important when resistance is still rare and encoded by more than one locus. This is because the establishment of spontaneously emerged resistant strains in such a population is delayed (Escalante *et al*., 2009). In low transmission settings, the scenario is exactly the opposite: there is less recombination and a more clonal population structure due to inbreeding. Therefore, the establishment of resistant strains in such a population is quick (Escalante *et al*., 2009). With the current state of global travel today, it is simple for resistance from an established source of resistance outside of Uganda and Africa to easily be introduced into the country (Uganda) or the continent (Africa).

In Africa, Artemisinin agents are used mainly as combination therapy whereas studies have shown that up to 78 % of their use in Cambodia where resistance has been reported are as monotherapy (Winzeler & Manary, 2014). The use of artemisinin agents in combination with other drugs may be conferring protection to the African population against emergence of parasites resistant to artemisinin (Meshnick, Taylor, & Kamchonwongpaisan, 1996). Meanwhile in areas where artemisinin monotherapy has been widely used like Guyana and Senegal, the practice has been associated with selective pressure for emergence of mutations in the K13 propeller gene of *P. falciparum* parasites (Jambou *et al*., 2005). Uganda recently also adopted the use of artemisinin monotherapy (IV artesunate) in treatment of complicated malaria (Li & Weina, 2010) and this could enhance the potential for emergence of resistant parasites.

In the current study, the observation was that there were no parasites present in the blood samples of patients screened on day seven (7) after completing a full dose of AL antimalarial

treatment. There are various factors that could account for this observation and one possible explanation could be that the medication is highly effective in this setting as parasites were cleared from the host completely. This has been backed by studies that have reported that artemisinin agents are still highly effective in clearing parasites promptly in Uganda (Conrad *et al*., 2014; Lin & Zaw, 2015) and other parts of Africa (Lin & Zaw, 2015).

Another possible explanation for the absence of *Plasmodium* parasites in patients' blood on day seven could be that the natural immunity within these study participants could be sufficient to clear remaining parasites from circulation after therapy with an artemisinin agent. Even though mutations exist in the K13 propeller gene of parasites isolated from some study participants, the high possibility of these strains being polyclonal would render them susceptible to a combination of artemisinin pressure and a high natural immunity within these study participants. There is the fact that malaria is an endemic disease in Uganda with high transmission intensity of parasites within host populations. Therefore, with constant exposure of hosts to the *Plasmodium* parasites, the host's body overtime builds up a natural immunity against *Plasmodium* parasites (Djimde *et al*., 2003). Hence in case of infection with *Plasmodium*, the host body's immune system is able to mount a sufficient response to prevent progression of the disease from the point of inoculation and also to clear remaining parasites after antimalarial therapy. A study by Djimde *et al.*, 2003, showed that the host body's immune system is able to ensure that all *Plasmodium* parasites are completely eliminated from the host body after therapy (Djimde *et al*., 2003). In the current study, the detected of mutations in some parasites did not correlate with their persistence in hosts up to day seven. This could be attributed to the fact that these mutations are not associated with resistance as observed from other studies (Taylor *et al*., 2014; Imwong *et al*., 2017; Nyunt *et al*., 2017).

Currently there is no known effective alternative medicine to artemisinin based antimalarial agents for malaria treatment globally. Therefore, if resistance to the current widely used artemisinin agents is to occur, then the risk of morbidity and mortality from malaria especially in endemic areas of the world is expected to increase.

CHAPTER SIX: CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

P. falciparum K13-gene mutations whose role in causing artemisinin resistance is not known were detected among *P. falciparum* parasites isolated from patients attending care at Kasangati Health Center IV, Wakiso district in central Uganda. There were no *Plasmodium* parasites detected in patients on day 7 after taking full dose of AL treatment which could be a possible indicator of the high efficacy of these agents in malaria treatment in Uganda.

6.2 Recommendations

- AL medication be administered only after confirmation of malaria unless otherwise. Medication use without confirmatory diagnosis can lead to inappropriate use of medication and hence drive the emergence of resistance.
- Patients on AL treatment need to be regularly followed up to assess adherence, re-assess antimalarial treatment success so as to help monitor and track resistance development.
- Findings from the current study also recommend that *P. falciparum* clearance half-life be monitored closely so as to give a better picture of the effect of mutations in the K13 gene.
- From the current study findings, it is recommended that monitoring of resistance to artemisinin based antimalarials be carried out on a regular basis.

6.3 Limitations of the study

- Patients recruited into the study were followed up for a short period of time as it is recommended that patients be followed for not less than 28 days. This is because there is a possibility that the parasite can remain dormant cause disease after residual medication has been flushed out of the body.
- The method used to assess presence of parasites on day seven (microscopy) was not very sensitive. More sensitive methods such as PCR would have been preferred.
- The current study did not ascertain whether the patients presenting to the health facility had taken any malaria medications before seeking medical care. This could affect the

study results in that parasite clearance could be augmented by the previous medication thereby masking the effects of observed mutations.

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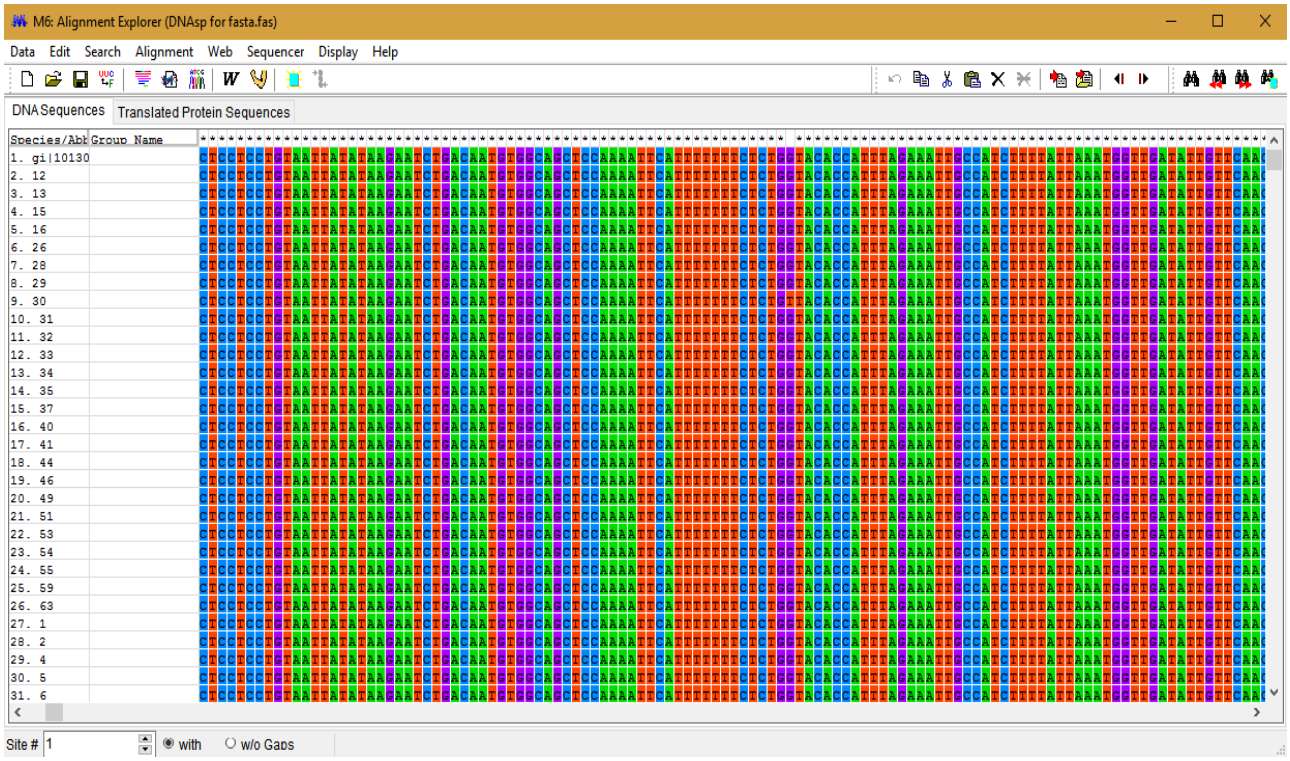
APPENDIX 1: Image of aligned data in MEGA



APPENDIX 2: Images of different *Plasmodium* strains observed under a microscope (100x)