DIFFERENTIAL PREVALENCE OF *PLASMODIUM FALCIPARUM* TRANSPORTER POLYMORPHISMS AND INFECTION COMPLEXITY IN CHILDREN WITH SYMPTOMATIC AND ASYMPTOMATIC MALARIA IN TORORO, UGANDA

BY

TUKWASIBWE STEPHEN

2010/HD17/1441U

BLT (Mak)

A DISSERTATION SUBMITTED TO SCHOOL OF GRADUATE STUDIES IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN MOLECULAR BIOLOGY AND BIOTECHNOLOGY OF MAKERERE UNIVERSITY

SEPTEMBER, 2013
DECLARATION

I Stephen Tukwasibwe do declare that this research dissertation is my original work and to the best of my knowledge, it has never been published or submitted for any other degree award to any university.

Signature…………………………………………………Date……………………………………

This research dissertation has been submitted for examination with the approval of the following supervisors:

1. Dr. Samuel Nsobya Lubwama. Msc, PhD

Signature…………………………………………………Date……………………………………

Senior Lecturer, Department of Pathology
Makerere University, College of Health Sciences

2. Associate Prof. Moses Joloba. MBChB, Msc, PhD

Signature…………………………………………………Date……………………………………

Head, Department of Microbiology
Makerere University, College of Health Sciences

3. Dr. Anne Kazibwe. Msc, PhD

Signature…………………………………………………Date……………………………………

Department of Veterinary Parasitology and Microbiology
College of Veterinary Medicine, Animal resources and Biosecurity
DEDICATION

This work is dedicated to my mother Mrs. Katuramu Nonsiata, my brothers and sisters, and my Uncle Mr. Kapere Richard.
ACKNOWLEDGEMENTS

I would like to gratefully acknowledge the enthusiastic supervision of Professor Philip J Rosenthal, Dr Samuel L Nsobya, Dr. Moses L Joloba and Dr. Anne Kazibwe. I would also like to thank the study team and participants of ACT PRIME project. Furthermore, I acknowledge the sponsor of this project, Fogarty International Center. Lastly, I sincerely appreciate the staff of Molecular Research Laboratory in Mulago who assisted me to finish this project in time.
Table of Contents
DECLARATION...........................................................................................................................................ii
DEDICATION..................................................................................................................................................iii
ACKNOWLEDGEMENTS............................................................................................................................iv
LIST OF FIGURES...........................................................................................................................................vii
LIST OF TABLES............................................................................................................................................viii
LIST OF ABBREVIATIONS AND ACRYNOYMS.........................................................................................ix
ABSTRACT.....................................................................................................................................................xi
CHAPTER ONE ............................................................................................................................................. 1
INTRODUCTION .......................................................................................................................................... 1
1.1 Background ........................................................................................................................................... 1
1.2 Problem statement ............................................................................................................................... 3
1.3 Significance ......................................................................................................................................... 4
1.4 Objectives of the study ....................................................................................................................... 4
The specific objectives were: .................................................................................................................. 4
1.5 Hypotheses ......................................................................................................................................... 4
CHAPTER TWO ........................................................................................................................................... 5
LITERATURE REVIEW ............................................................................................................................. 5
2.1 Malaria epidemiology ........................................................................................................................ 5
2.2 Malaria transmission ........................................................................................................................ 6
2.3 Antimalarial drug resistance ............................................................................................................ 8
2.4 Plasmodium falciparum chloroquine transporter (pfcrt) gene ...................................................... 8
2.5 Plasmodium falciparum multi-drug resistance (pfmdr 1) gene ..................................................... 9
2.6 Complexity of infection ................................................................................................................... 10
2.7 Association of P. falciparum transporter polymorphisms with clinical outcome ...... 11
CHAPTER THREE ..................................................................................................................................... 13
MATERIALS AND METHODS ................................................................................................................ 13
3.1 Study design ..................................................................................................................................... 13
3.2 Sampling criteria.............................................................................................................................. 14
3.3 Study sample size ............................................................................................................................ 14
3.4 Molecular studies.................................................................................................................. 14
3.4 1 DNA extraction.................................................................................................................. 14
3.4 2 Detection of polymorphisms at pfmdr 1 alleles N86Y and D1246Y ......................... 15
3.4 3 Detection of the pfcr t K76T Polymorphisms ................................................................. 16
3.4 4 Complexity of Infection.................................................................................................. 17
3.5 Ethical approval.................................................................................................................... 17
3.6 Statistical analysis............................................................................................................... 17
CHAPTER FOUR ...................................................................................................................... 18
RESULTS .................................................................................................................................. 18
4.1 Characteristics of study participants.................................................................................. 18
4.2 Prevalence of Plasmodium falciparum transporter (pfcr t and pfmdr1) polymorphisms ................................................................................................................................. 18
4.3 Association of transporter polymorphisms mediating resistance to antimalarial drugs and complexity of infection with clinical presentations........................................... 21
4.4 Complexity of plasmodium falciparum infections in symptomatic documented fever, symptomatic reported fever and asymptomatic malaria................................................. 22
CHAPTER FIVE .......................................................................................................................... 25
DISCUSSION .............................................................................................................................. 25
5.1 Discussion ............................................................................................................................ 25
CHAPTER SIX ............................................................................................................................ 29
CONCLUSION AND RECOMMENDATIONS ......................................................................... 29
6.1 Conclusion ........................................................................................................................... 29
6.2 Recommendations ............................................................................................................. 29
REFERENCES .............................................................................................................................. 30
APPENDICES .......................................................................................................................... 38
LIST OF FIGURES

Figure 1: Prevalence of pfcrK76T mutations in symptomatic documented fever, symptomatic reported fever and asymptomatic malaria……………………………………..23

Figure 2: Prevalence of pfmdr1 N86Y mutations in symptomatic documented, symptomatic reported and asymptomatic malaria………………………………………………24

Figure 3: Prevalence of pfmdr1 D1246Y mutations in symptomatic documented, symptomatic reported and asymptomatic malaria. ………………………………………………………...25

Figure 4: A photograph showing a representative 2.5% agarose gel for 3D7 allele………27

Figure 5: A photograph showing a representative 2.5% agarose gel for FC27 allele…..28

Figures 6: Association of mean infection complexity with clinical presentation…………29
LIST OF TABLES

Table 1: PCR primers for the detection of \textit{Pfmdrl} N86Y and D1246Y Polymorphisms…16

Table 2: PCR primers for the detection of \textit{Pfcrt} K76T polymorphisms.........................17

Table 3: Characteristics of study participants.................................................................21

Table 4: Risk factors associated with symptomatic malaria...........................................26
### LIST OF ABBREVIATIONS AND ACRONYMS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT</td>
<td>artemisinin-based combination therapy</td>
</tr>
<tr>
<td>AL</td>
<td>artemether-lumefantrine</td>
</tr>
<tr>
<td>AS/AQ</td>
<td>arsunate-amodiaquine</td>
</tr>
<tr>
<td>COI</td>
<td>Complexity of infections</td>
</tr>
<tr>
<td>CQ</td>
<td>chloroquine</td>
</tr>
<tr>
<td>CQR</td>
<td>chloroquine resistance</td>
</tr>
<tr>
<td>DHA</td>
<td>dihydroartemisinin</td>
</tr>
<tr>
<td>DHA/PQ</td>
<td>dihydroartemisinin/piperaquine</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>GEE</td>
<td>Generalized Estimated Equation</td>
</tr>
<tr>
<td>ml</td>
<td>mililitre</td>
</tr>
<tr>
<td>msp-2</td>
<td>Merozoite Surface Protein- 2</td>
</tr>
<tr>
<td>MT</td>
<td>Mutant</td>
</tr>
<tr>
<td>OR</td>
<td>Odds Ratio</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Pfcrt</td>
<td><em>Plasmodium falciparum</em> chloroquine resistance gene</td>
</tr>
<tr>
<td>Pfmdr1</td>
<td><em>Plasmodium falciparum</em> multidrug resistance gene</td>
</tr>
<tr>
<td>QN</td>
<td>quinine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>Rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SNPs</td>
<td>Single nucleotide polymorphisms</td>
</tr>
<tr>
<td>SP</td>
<td>sulfadoxine and pyrimethamine</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
<tr>
<td>μl</td>
<td>microlitre</td>
</tr>
</tbody>
</table>
ABSTRACT

Malaria, particularly infection with *Plasmodium falciparum*, remains one of the most important infectious diseases in the world. A major challenge to the treatment and control of malaria has been resistance to most available antimalarial drugs. Polymorphisms in *pfcrt* and *pfmdr1*, genes encoding putative drug transporters, impact upon sensitivity of *P. falciparum* to multiple drugs. Considering the strong and, at times, reciprocal pressures of antimalarial drugs on parasite genetics and the impact of transporter polymorphisms on sensitivity to important ACT components. This study was aimed at determining the differential prevalence of *P. falciparum* transporter polymorphisms and complexity of infection in children with symptomatic and asymptomatic malaria in Tororo, Uganda. Two hundred and forty three children with documented fever (cases), and equal numbers randomly selected from the other clinical categories (controls) were studied. A multivariate analysis adjusting for age, COI, and parasite density was conducted, accounting for clustering with generalized estimating equations models fitted for each polymorphism, with robust standard errors.

The prevalence of wild type genotypes was significantly higher in febrile compared to asymptomatic malaria infected children. There was no wild type genotype detected for *pfcrt* gene. *Pfcr* mixed genotypes were significantly higher in febrile compared to asymptomatic malaria infected children. These results indicate that parasites with wild type genotypes associated with decreased sensitivity to both components of the Ugandan first-line malaria treatment artemether-lumefantrine were more likely than those with mutant genotypes to be associated with symptomatic malaria infection.
CHAPTER ONE

INTRODUCTION

1.1 Background

Malaria, particularly infection with *Plasmodium falciparum*, remains one of the most important infectious diseases in the world resulting in 219 million cases and an estimated 660,000 deaths annually worldwide (WHO, 2010). In Sub Saharan Africa, it has been reported that a child dies of malaria disease every minute (WHO, 2010). The major challenge to malaria treatment and control has been resistance to most available antimalarial drugs. In light of this problem, artemisinin-based combination therapy (ACT), including a potent and rapid-acting artemisinin (artesunate, artemether, or dihydroartemisinin) and a longer-acting partner drug (lumefantrine, amodiaquine, piperaquine, or mefloquine), is now the standard of care for the treatment of falciparum malaria.

In Uganda, artemether lumefantrine is the first line antimalarial therapy and artemisinin/piperaquine an alternative treatment. Many ACTs efficacious studies have been done and the data shows the drugs are still very efficacious (Yeka, Tibenderana, Achan, D'Alessandro, & Talisuna, 2013). However, resistance to artemisinins, manifested as delayed parasite clearance after therapy, is increasing in parts of Southeast Asia, and resistance has been seen to most artemisinin- partner drugs (Dondorp *et al.*, 2009; Noedl *et al.*, 2008).

Altered sensitivity to a number of drugs is mediated in part by polymorphisms in *pfcrt* and *pfmdr1*, genes encoding two putative *P. falciparum* transporter proteins. The *pfcrt* K76T mutation is the major mediator of resistance to chloroquine and amodiaquine (Djimde *et al.*, 2001).
Considering the strong and, at times, reciprocal pressures of antimalarial drugs on parasite genetics, and the impacts of transporter polymorphisms on sensitivity to important ACT components, it is of interest to determine the effects of transporter polymorphisms on parasite fitness and virulence. Considering fitness, valuable insight has come from experiences in areas where widespread chloroquine resistance led to discontinuation of the drug. In Malawi and Hainan Island, China, discontinuation of chloroquine for the treatment of malaria led to dramatic changes in circulating parasites, with the return of chloroquine sensitive, pfcr\textit{t} wild type parasites and also of strong antimalarial efficacy for chloroquine (Laufer \textit{et al.}, 2010; Wang \textit{et al.}, 2005). Clearly, chloroquine-sensitive parasites have a fitness advantage over resistant parasites. For polymorphisms in \textit{pfmdr1}, in \textit{in vitro} competitive growth experiments, wild type parasites had a fitness advantage over those with 3 polymorphisms, only one of which (1246Y) is common in Africa (Hayward, Saliba, & Kirk, 2005). When mixed clinical isolates were followed in culture, modest fitness advantages appeared associated with the mutant 86Y and wild type D1246 alleles (Ochong, Tumwebaze, Byaruhanga, Greenhouse, & Rosenthal, 2013).

Comparisons of parasites circulating during high and low transmission seasons showed the prevalence of parasites with mutant \textit{pfcr\textit{t}} 76T and \textit{pfmdr1} 86Y sequences to decrease during the low transmission season, when drug pressure is lowest, implying a fitness advantage for wild type parasites (Ord \textit{et al.}, 2007). Overall, although measures of parasite fitness are limited and imperfect, in most cases parasites with wild type sequences in \textit{pfcr\textit{t}} and \textit{pfmdr1} have appeared to have a fitness advantage over mutant parasites. It is not clear if parasites with resistant or sensitive genotypes differ in their abilities to cause disease. To further explore associations between \textit{P. falciparum} resistance-mediating polymorphisms and parasite fitness or virulence,
differences in genotypes between parasites causing symptomatic and asymptomatic infections in Uganda were studied.

In malaria endemic areas, one individual can be simultaneously infected with multiple *P. falciparum* strains (Nsobya, Kiggundu, Joloba, Dorsey, & Rosenthal, 2008). Such multiple strain infections, complexity of infections (COI) can be an indicator of the immune status of an individual. (al-Yaman et al., 1997). To further explore the association of complexity of infection with clinical malaria in a malaria endemic area, differences in mean complexity of infection in children with symptomatic and asymptomatic malaria in Tororo, an area with high malaria transmission intensity in Uganda were studied.

### 1.2 Problem statement

Single nucleotide polymorphisms in *pfcr* and *pfmdrl*, genes encoding putative drug transporters, impact upon sensitivity of *P. falciparum* to multiple drugs. In particular the *pfcr* 76T and *pfmdrl* 86Y and 1246Y mutations are associated with decreased sensitivity to chloroquine and amodiaquine, but increased sensitivity to artemisinins, lumefantrine, and mefloquine. In addition to drug resistance, complexity of infection is also high in malaria endemic areas. Therefore, resistance-mediating mutations and complexity of infection may impact upon parasite virulence, fitness and clinical presentation. No study has been conducted in Uganda to analyze the association of single nucleotides polymorphisms mediating resistance to antimalarials and complexity of infection with different malaria clinical presentations. Therefore this study was aimed at exploring differences in molecular markers mediating resistance to antimalarials and complexity of infection in *P. falciparum* in relation to symptomatic or asymptomatic malaria.
1.3 Significance

This study has helped to understand the impact of *P. falciparum* parasites factors (genotypes and infection complexity) to different malaria clinical presentations in malaria endemic populations. Results from this study are useful in determining *P. falciparum* genotypes and mean infection complexity that is likely to be associated with clinical malaria. This information is useful in laying strategies for the development of an effective malaria vaccine.

1.4 Objectives of the study

The general objective was to determine the differential prevalence of transporter polymorphisms and complexity of infection in symptomatic and asymptomatic falciparum malaria infections in Tororo, Uganda.

The specific objectives were:

1. To determine the prevalence of single nucleotides polymorphisms mediating resistance to antimalarials among *P. falciparum* transporter genes in children with different malaria clinical presentations in Tororo, Uganda.

2. To determine the complexity of infection of *Plasmodium falciparum* using merozoite surface protein 2 in children with different malaria clinical presentations in Tororo, Uganda.

1.5 Hypotheses

1. *Plasmodium falciparum* strains with wild type genotypes are more likely to cause symptomatic malaria.

2. Symptomatic malaria infected children have higher complexity of infection of *Plasmodium falciparum* strains compared to asymptomatic malaria infected children.
CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria epidemiology

Malaria is a life-threatening disease caused by parasites that are transmitted to people through the bites of infected anopheles mosquitoes. Increased malaria prevention and control measures are dramatically reducing the malaria burden in many places. Children 6 months to 5 years, non immune pregnant women, and international travellers from non endemic areas are very vulnerable to the disease when they get infected. According to the World Health Organization’s malaria report, 2010, 219 million malaria cases were reported worldwide with 78% of the cases in Africa with estimated 660,000 deaths.

Globally, 91% of malaria deaths occurred in Sub-Saharan Africa of which 85% occurred in children below 5 years. Uganda is ranked the sixth worldwide in the number of malaria cases and third in number of malaria deaths (WHO, 2010). Malaria causes significant morbidity, mortality and economic loss in malaria endemic countries (Sachs & Malaney, 2002). Hospital records show that malaria is responsible for 30% to 50% of outpatient visits, 15% to 20% of admissions and 9% to 14% of inpatient deaths (WHO, 2010). Africa has several factors that make it high risk for malaria. Some of these include very efficient mosquito species (Anopheles gambiae) responsible for transmission, predominant parasite species (Plasmodium falciparum) that leads to more severe malaria, warm and humid climate that allows transmission to occur year round as well as lack of resources and poor socio-economic conditions that prevents malaria control efforts (WHO, 2010). Other areas that are at risk include some countries in South America and South Asia (WHO, 2010). Early diagnosis and treatment of malaria reduces disease and prevents deaths. It also contributes to reducing malaria transmission. The best available treatment,
particularly for *P. falciparum* malaria, is artemisinin-based combination therapy (Yeka et al., 2005).

2.2 Malaria transmission

Malaria is transmitted exclusively through the bites of *Anopheles* mosquitoes. The intensity of transmission depends on factors related to the parasite, the vector, the human host, and the environment. About 20 different *Anopheles* species are locally important around the world. All of the important vector species bite at night. *Anopheles* mosquitoes breed in water and each species has its own breeding preference; for example some prefer shallow collections of fresh water, such as puddles, rice fields, and hoof prints.

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) and where it prefers to bite humans rather than other animals. For example, the long lifespan and strong human-biting habit of the African vector species is the main reason why more than 90% of the world's malaria deaths are in Africa. Transmission also depends on climatic conditions that may affect the number and survival of mosquitoes, such as rainfall patterns, temperature and humidity. In many places, transmission is seasonal, with the peak during and just after the rainy season.

Malaria transmission is not homogeneous throughout an endemic area but spotty and depends on the location of mosquito breeding sites and the clustering of human habitations where people serving as reservoirs of parasites for mosquito infection live. Stable malaria implies that the prevalence of the infection is sufficiently high to engender a substantial level of clinical immunity within a population. In contrast, un stable malaria is characterized by spatial, temporal variability and is associated with lower levels of clinical immunity within the population.
Asymptomatic parasitaemia is prevalent in highly endemic areas of Africa, reaching over 90% in children (Bottius et al., 1996).

Single nucleotides polymorphisms mediating resistance to antimalarials may be playing a role in the development of clinical malaria but this is not fully understood. It is assumed that in endemic areas asymptomatic parasitaemia is involved in the development of partial immunity (Staalsoe & Hviid, 1998) and may protect against clinical disease from new infections (Farnert et al., 1999). Asymptomatic parasitaemia also provides a reservoir for transmission and may be a precursor in the progression to symptomatic disease. Asymptomatic *Plasmodium falciparum* infections of varying duration and resolving without therapy have also been reported (Missinou, Lell, & Kremsner, 2003). A high rate of asymptomatic *P. falciparum* prevalence has been well known to occur in areas of high malaria transmission in Africa and other parts of the world. Asymptomatic *Plasmodium falciparum* carriers had a higher parasite density and a lower probability of developing malaria attack during the subsequent rainy season than did none carriers in Senegalese children living in areas where malaria was endemic with seasonal transmission (Males, Gaye, & Garcia, 2008). These results suggest that in malaria endemic areas, asymptomatic carriers of *Plasmodium falciparum* may have some degree of protection against clinical malaria or malaria attack in the subsequent malaria transmission season. This called for a study to elucidate why asymptomatic *Plasmodium falciparum* carriers do not develop clinical malaria.

In a study that was carried out in western Kenya, a malaria endemic area, there was no significant difference in the frequencies of *P. falciparum* mutations between symptomatic and asymptomatic malaria infected primary school children aged 6-14 years (Zhong et al., 2008). These results may have missed detecting association among the two groups because these
children were not stratified according to age groups when the analysis was being done and yet partial immunity to malaria varies with age.

2.3 Antimalarial drug resistance

Resistance of *Plasmodium falciparum* to the available drugs remains a major challenge to the control of malaria. Older drugs, including the aminoquinolines (chloroquine (CQ) and amodiaquine (AQ)) and antifolates (sulfadoxine/pyrimethamine (SP)) are already seriously compromised, with unacceptable levels of treatment failure in most parts of Africa and (Wongsrichanalai, Pickard, Wernsdorfer, & Meshnick, 2002) In areas where cases of drug resistance to antimalarials are high, WHO has recommended artemisinin based combination therapy (ACT) for the treatment of uncomplicated falciparum malaria (Nosten & White, 2007). The commonly used ACTs in Africa are artemether/lumefantrine, artesunate/ amodiaquine (AS/AQ), and dihydroartemisinin/piperaquine (DHA/PQ), each containing artemisinin derivatives combined with a longer-acting drug. These ACTs have shown excellent efficacy for the treatment of malaria in Africa. However, there is concern that heavy use of ACTs will offer strong selective pressure for parasites with decreased sensitivity to the drugs. This may seriously affect the efficacy of ACTs in areas where they have been effective. Resistance cases of ACTs have been reported in Southeast Asia (Dondorp *et al.*, 2009; Noedl *et al.*, 2008), though not yet well characterized.

2.4 *Plasmodium falciparum* chloroquine transporter (*pfcrt*) gene

The *pfcrt* gene located on chromosome 7 of *Plasmodium falciparum* has 13 exons and encodes an integral membrane protein located on the membrane of the intra-erythrocytic parasite's digestive vacuole. It is a member of a drug transporter super family (Martin & Kirk, 2004; Tran & Saier, 2004). Twenty point mutations in the *pfcrt* gene to date have
been associated with chloroquine resistance in the field (Cooper et al., 2005). Studies have shown that substitution of threonine (T) for lysine (K) at position 76 (K76T) is the main cause of chloroquine resistance (CQR) in *P. falciparum* parasites. In a study, done in Uganda, the prevalence of the 76T mutation was 100% (Dorsey, Kamya, Singh, & Rosenthal, 2001).

There are two haplotypes of *pfcrt* gene; CVIET from Asian and African isolates and SVMNT from South America which have been associated with CQ resistance (Cortese, Caraballo, Contreras, & Plowe, 2002; Vieira et al., 2004). Additional studies (Dittrich et al., 2005) reported that the SVMNT haplotype found in Southeast Asia appeared to be associated with a decreased efficacy of amodiaquine (AQ).

### 2.5 Plasmodium falciparum multi-drug resistance (pfmdr 1) gene

The *pfmdr 1* gene, located on chromosome 5, encodes a predicted 12-transmembrane domain protein, known as P glycoprotein homologue (Pgh-1) a member of the ABC transporter family (Foote & Kemp, 1989). The gene *Pfmdr 1* is a homologue of mammalian P glycoprotein and shares common features which are the determinants of multi drug resistance in mammalian tumor cells (Duraisingh & Refour, 2005). The P glycoprotein homologue is located on the parasite digestive vacuole, which is also the site of action of CQ and possibly for other quinoline-based antimalarial drugs, including amodiaquine (AQ) and Quinine (QN) (Foley & Tilley, 1998) and (O'Neill, Bray, Hawley, Ward, & Park, 1998). Five different mutations in *pfmdr 1* have been associated both *in vivo* and *in vitro* with drug resistance to quinine, halofantrine and artemisinin derivatives: N86Y, F184Y, S1034C, N1042D, and D1246Y (Duraisingh et al., 2000; Wongsrichanalai et al., 2002). Recent studies (Dokomajilar, Nsobya, Greenhouse, Rosenthal, & Dorsey, 2006; Sisowath et al., 2005) have also reported that wild
type \textit{pfmdr} 1 86N is selected for by prior therapy with Artemether Lumefantrine although the drug is still efficacious.

\textbf{2.6 Complexity of infection}

Complexity of infection (COI) of \textit{Plasmodium falciparum} is the number of different \textit{Plasmodium falciparum} strains co infecting a single host (Vafa, Troye-Blomberg, Anchang, Garcia, & Migot-Nabias, 2008). The complexity of infection can be determined by PCR-based genotyping of highly polymorphic merozoite surface protein-2 (msp-2) gene (Kyabayinze \textit{et al.}, 2008). In malaria endemic areas like Tororo in Uganda, COI can be a useful indicator of the transmission level (Babiker, Ranford-Cartwright, & Walliker, 1999). Data suggests that the average number of malaria parasites strains in an individual is well correlated to the transmission level (Arnot, 1998).

Some studies reported that a higher COI was associated with increased risk to subsequent clinical malaria (al-Yaman \textit{et al.}, 1997). Others reported that a high COI was associated with protection against infection (Contamin \textit{et al.}, 1996). In addition to indicating host susceptibility to malaria transmission, the COI might impact the development of immunity in either a positive or a negative way. Infection with different genotypes might lead to the development of genotype-specific or allele-specific immunity. If this occurs, a host would only develop resistance to an immunologically defined genotype, remaining susceptible to others (Gupta & Day, 1994). Multiple parasite strain infections play an important role in the development of strain specific immunity (Kyabayinze \textit{et al.}, 2008). Individuals may develop immunity to infection from some but not all strains to which they are exposed. No data is available to show the number of parasite strains (COI) in a malaria endemic area like Tororo in Uganda that can result in the development of clinical malaria. Therefore, there was the need for a study to investigate the number of \textit{P. falciparum} strains likely to predict clinical outcome.
2.7 Association of \textit{P. falciparum} transporter polymorphisms with clinical outcome

Considering that antimalarial drugs pressure and transporter polymorphisms impact on parasite genetics and sensitivity to important ACT components, it is of interest to determine their effects on parasite fitness and virulence. For instance, in areas where widespread chloroquine resistance led to discontinuation of the drug in Malawi and Hainan Island, China, discontinuation of chloroquine for the treatment of malaria led to dramatic changes in circulating parasites, with the return of chloroquine sensitive, \textit{pfcrt} wild type parasites and also of strong antimalarial efficacy for chloroquine (Laufer \textit{et al.}, 2010). Clearly, chloroquine-sensitive parasites have a fitness advantage over resistant parasites. For polymorphisms in \textit{pfmdr 1}, \textit{in vitro} competitive growth experiments, wild type parasites had a fitness advantage over those with 3 polymorphisms, only one of which (1246Y) is common in Africa (Hayward \textit{et al.}, 2005). When mixed clinical isolates were followed in culture, modest fitness advantages appeared associated with the mutant 86Y and wild type D1246 alleles (Ochong \textit{et al.}, 2013).

Comparisons of parasites circulating during high and low transmission seasons showed the prevalence of parasites with mutant \textit{pfcrt} 76T and \textit{pfmdr 1} 86Y sequences to decrease during the low transmission season, when drug pressure is lowest, implying a fitness advantage for wild type parasites (Ord \textit{et al.}, 2007). Overall, although measures of parasite fitness are limited and imperfect, in most cases parasites with wild type sequences in \textit{pfcrt} and \textit{pfmdr 1} have appeared to have a fitness advantage over mutant parasites. The prevalence of the \textit{pfcrt} 76T mutation was very high, and unchanged between symptomatic and asymptomatic children in Benin (Ogouyemi-Hounto \textit{et al.}, 2013). Parasites harboring the \textit{pfcrt} 76T, \textit{pfmdr 1} 86Y, and \textit{pfmdr 1} 1246Y mutations have decreased sensitivity to chloroquine and amodiaquine, but increased sensitivity to other important antimalarials, including artemisinins, lumefantrine, and
mefloquine. To further explore associations between *P. falciparum* resistance-mediating polymorphisms and parasite fitness or virulence, there was need for a study to determine differences in genotypes between parasites causing symptomatic and asymptomatic infections in Ugandan children.
CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design

This was an unmatched, case control study designed to determine associations between *P. falciparum* drug resistance genotypes and presentation of children with symptomatic documented fever, symptomatic reported fever and asymptomatic malarial infections. Children < 15 years suffering from symptomatic documented fever (cases) and symptomatic reported fever or asymptomatic malaria (controls) infections were studied by comparing the prevalence of *P. falciparum* transporter (*pfcr* and *pfmdr1*) polymorphisms and complexity of infection.

This study utilized samples from the ACT PRIME study, a cluster-randomized community survey that was conducted in Tororo district, Uganda an area with high malaria transmission intensity. Briefly, the ACT PRIME study evaluated the impact of enhanced health facility based care for malaria and febrile illnesses in children compared to the standard care provided by the government. A component of this project was a cross sectional study of 8798 children who were evaluated by collection of clinical history (presence or absence of fever, chills, vomiting, sweating) and measuring body temperatures by the clinician using a clinical thermometer and collection of blood by finger stick for blood smears and storage on FTA filter papers for molecular studies.

Evaluation for polymorphisms at the *pfcr* K76T, *pfmdr1* N86Y and *pfmdr1* D1246Y alleles and complexity of infection from the FTA filter paper samples collected by the ACT PRIME study team was carried out between June 2010 and June 2012. This study utilized samples from 4392
children with parasitemia from 20 clusters. Clusters were defined as health facilities and their catchment areas. These included households within 20 km radius of the health centre.

3.2 Sampling criteria

Only children with a positive blood smear for *p. falciparum* were considered. Children with a positive blood smear were categorized as asymptomatic (afebrile without report of fever or other illnesses), fever by report (afebrile but history of fever within the last 48 hours) and documented fever (temperatures ≥ 38°C at evaluation).

3.3 Study sample size

Samples for analysis were from all the 243 children with symptomatic documented fever (cases) and equal numbers were randomly selected from each of the symptomatic reported and asymptomatic malaria infected children (controls).

3.4 Molecular studies

3.4.1 DNA extraction

*Plasmodium falciparum* genomic DNA was isolated by Chelex extraction (Plowe & Wellems, 1995). Briefly, blood spots of approximately 6 mm in diameter were punched into 1.5 ml microfuge tubes and soaked in 1 ml of 1X phosphate buffered saline (PBS) and 50μl of 10% saponin. These microfuge tubes were inverted several times and stored overnight at 4°C. The microfuge tubes were centrifuged at 13,000 rpm for 5 seconds and the PBS/saponin aspirated. After discarding the supernatant, 1ml of 1X PBS (no saponin) was added and the microfuge tubes were inverted several times and then incubated at 4°C for 30 minutes. The microfuge tubes were centrifuged at 13,000 rpm for two minutes to wash the blood spots. After discarding the supernatant, 100μl of sterile water and 50μl of vortexed chelex stock solution was dispensed into
each of the microfuge tubes containing blood spots. Parasite DNA was extracted by incubating
the microfuge tubes for 10 minutes on a 95°C heat block coupled with vigorous vortexing of
each sample after two minutes during the incubation. After incubation, microfuge tubes were
centrifuged for 5 minutes at high speed and 200 μl was transferred into a clean microfuge tube
which was then centrifuged for 10 minutes and the final white to yellow supernatant (DNA) was
transferred to another labeled microfuge tube and was stored at -20°C.

3.4.2 Detection of polymorphisms at pfmdr 1 alleles N86Y and D1246Y

To analyze for polymorphisms at codons 86 and 1246 of the pfmdr 1 gene, flanking sequences
were amplified by nested PCR (Appendix 111 for the master mix reaction) and digested with
specific restriction enzymes (Duraisingh et al., 2000). See Table 1 for primers

Table 1: PCR primers for the detection of pfmdr 1 N86Y and D1246Y Polymorphisms

| Pfmdr 1 86 first round | MDR-A1:(30bp) (5’-TGT TGA AAG ATG GGTA AAG AGC AGA AAG AG-3’)  
|                       | MDR-A3:(33bp) (5’-TAC TTT CTT ATT ACA TAT GAC ACC ACA AACG-3’) |
| Pfmdr 1 86 second round | MDR-A4: (30bp) (5’-AAA GAT GGT AAC CTC AGT ATC AAA GAA GAG -3’)  
|                       | MDR-A2:(33bp) (5’- GTC AAA CGT GCA TTT TTT ATT ATT GAC CAT TTA-3’) |
| Pfmdr 1 1246 first round | MDR-01 :(33bp) (5’-AGA AGA TTA TTT CTG TAA TTT GAT ACA AAA AGC-3’)  
|                       | MDR-02:(30bp) (5’-ATG ATT CGA TAA ATT CAT CTA TAG CAG CAA-3’) |
| Pfmdr 1 1246 second round | 1246F:(35bp) (5’-ATG ATC ACA TTA TAT TAA AAA ATG ATA TGA CAA AT3’)  
|                       | MDR-02 :(30bp) (5’-ATG ATT CGA TAA ATT CAT CTA TAG CAG CAA-3’) |


Restriction enzymes Afl 111 and Bgl 11 were used to detect polymorphisms at codons 86 and
1246 of the pfmdr 1 gene at 37°C for 1 hour and 30 minutes respectively (Duraisingh, Curtis, &
Warhurst, 1998). Genomic DNA of FCR3 and 7G8 laboratory strains were used as positive
controls for wild type and mutants respectively. Distilled water was used as a negative control.
Controls were concurrently amplified, digested and run alongside the samples on the 2.5%
agarose gels and the results were classified as wild type, mutant or mixed based on migration patterns of the ethidium bromide stained fragments (560 bp for wild type fragments and 232 bp or 328bp for the mutant fragments).

3.4.3 Detection of the *pfcrt* K76T Polymorphisms

Polymorphism at codon 76 of *pfcrt* gene was determined using a PCR allele specific restriction enzymes analysis method (Djimde *et al.*, 2001). The sequences flanking codon 76 were amplified by PCR primers. See Table 2 for primers.

**Table 2: PCR primers for the detection of pfcrt K76T polymorphisms**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pfcrt 76 first round</strong></td>
<td>76-CRT1: <em>(5’GC GGCGCATGGCTACGTTAGTTGAGGAG)</em>’</td>
</tr>
<tr>
<td><strong>Pfcrt 76 second round</strong></td>
<td>76-CQA: <em>(5’TGTGCTATGTTAGTTAAAACTT)</em>’</td>
</tr>
</tbody>
</table>

From http://medschool.umaryland.edu/cvd/2002_pcr_asra.asp

Restriction enzyme Apo I (NEB) was used to detect Single nucleotide polymorphisms at codons 76 of *pfcrt* gene. Restriction enzyme digestion reaction was carried out at 50°C for 1 hour 30 minutes (Djimde *et al.*, 2001). Genomic DNA of HB3 and DD2 laboratory isolates were used as positive controls for wilt type and mutant respectively. Distilled water was used as a negative control. Controls were amplified, digested and run alongside the samples on 2.5% agarose gels. Restriction enzymes digestion products were examined by electrophoresis on 2.5% agarose gels in 1X Tris Acetate buffer and the results were classified as wild type, mutant or mixed based on migration patterns of the ethidium bromide stained fragments (34 bp or 100 bp for wild type fragments and 134 bp for the mutant fragments).
3.4.4 Complexity of Infection

Complexity of infection was analyzed by genotyping for *p. falciparum* 3D7 and FC27 alleles of Merozoite Surface Protein-2 (MSP-2) gene using nested PCR (Dokomajilar, Lankoande, *et al.*, 2006). 3D7 and HB3 laboratory strains were used as positive controls (standards). Nuclease free water was used as a negative control. PCR products were examined by electrophoresis on 2.5% agarose gels in 1X Tris Acetate buffer. The size of the product was compared to the standard on densitometric digitized gel images analyzed by GelCompar II software (Applied Maths). Each band represented an individual *P. falciparum* strain. Strains were considered identical if the fragment lengths difference was within 10 base pairs.

3.5 Ethical approval

The ACT PRIME study was approved by the Uganda National Council of Science and Technology, Makerere University Research and Ethics Committee, the London School of Hygiene and Tropical Medicine Institutional Review Board and the University of California, San Francisco Committee on Human Research. Parents/guardians were informed about the benefits of the study and written informed consent in the appropriate language was obtained from the parents or legal guardians (Appendix 1 and 11).

3.6 Statistical analysis

Data were entered and verified using Graph Pad Prism5 Demo software. Descriptive results including numbers and percentages were summarized using tables stratified by study groups (documented fever, reported fever and asymptomatic) and by clusters. A multivariate analysis adjusting for age, complexity of infection, and parasite density was conducted accounting for clustering with generalized estimating equations models fitted for each polymorphism, with robust standard errors. P-values less than 0.05 were considered statistically significant.
CHAPTER FOUR

RESULTS

4.1 Characteristics of study participants

The majority of the children were below five years (56%) and the rest were between 5 and 15 years (44%). Most children with documented fever were less than five years (67.9%).

Description of the baseline variables is as summarized in Table 3.

Table 3: Description of the baseline variables for study participants

<table>
<thead>
<tr>
<th></th>
<th>Documented fever</th>
<th>Reported fever</th>
<th>Asymptomatic</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>K76T Mutant</td>
<td>219 (90.1)</td>
<td>199 (81.9)</td>
<td>211 (86.8)</td>
<td>629 (86.3)</td>
</tr>
<tr>
<td>K76T Mixed</td>
<td>21 (8.6)</td>
<td>10 (4.1)</td>
<td>3 (1.2)</td>
<td>34 (4.7)</td>
</tr>
<tr>
<td>N86Y Wild type</td>
<td>115 (47.3)</td>
<td>67 (27.6)</td>
<td>47 (19.3)</td>
<td>229 (31.4)</td>
</tr>
<tr>
<td>N86Y Mutant</td>
<td>40 (16.5)</td>
<td>22 (9.0)</td>
<td>64 (26.3)</td>
<td>126 (17.3)</td>
</tr>
<tr>
<td>N86Y Mixed</td>
<td>88 (36.2)</td>
<td>119 (49.0)</td>
<td>118 (48.6)</td>
<td>325 (44.6)</td>
</tr>
<tr>
<td>D1246Y Wild type</td>
<td>24 (9.9)</td>
<td>8 (3.3)</td>
<td>7 (2.9)</td>
<td>39 (5.3)</td>
</tr>
<tr>
<td>D1246Y Mutant</td>
<td>72 (29.6)</td>
<td>90 (37.0)</td>
<td>86 (35.4)</td>
<td>248 (34.0)</td>
</tr>
<tr>
<td>D1246Y Mixed</td>
<td>111 (45.7)</td>
<td>108 (44.4)</td>
<td>138 (56.8)</td>
<td>357 (49.0)</td>
</tr>
<tr>
<td>Age group &lt; 5 years</td>
<td>165 (67.9)</td>
<td>121 (49.8)</td>
<td>122 (50.2)</td>
<td>408 (56.0)</td>
</tr>
<tr>
<td>Age group 5 – 15 years</td>
<td>78 (32.1)</td>
<td>122 (50.2)</td>
<td>121 (49.8)</td>
<td>321 (44.0)</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean COI</td>
<td>3.3 (1.7)</td>
<td>2.7 (1.4)</td>
<td>2.6 (1.4)</td>
<td>2.8 (1.5)</td>
</tr>
<tr>
<td>Parasite density</td>
<td>5400 (960 - 28400)</td>
<td>1700 (480 - 4960)</td>
<td>1040 (400 - 3600)</td>
<td>1800 (500 - 7820)</td>
</tr>
</tbody>
</table>

COI – complexity of infection, IQR – Inter Quartile Range, K76T, N86Y and D1246Y are *plasmodium falciparum* transporter polymorphisms at codons 76, 86 and 1246 of Pfcrt and Pfmdr1 respectively.

4.2 Prevalence of *Plasmodium falciparum* transporter (pfcrt and pfmdr1) polymorphisms

In this study, 243 samples were evaluated for each of the three different clinical presentations: symptomatic (documented fever), symptomatic (reported fever) and asymptomatic malaria, for single nucleotide polymorphisms mediating resistance in pfmdr1 genes (N86Y and D1246Y) and
*pfcr* genes (K76T). There was no *pfcr* K76T pure wild type in all samples analyzed from different clinical presentations. The prevalence of *pfcr* alleles with pure mutants in symptomatic (documented fever), symptomatic (reported fever) and asymptomatic malaria were: 219/240 (91.3%), 199/209 (95.2%) and 211/214 (98.6%) and for mixed infections (wild/mutant) were, 21/240 (8.7%), 10/209 (4.8%) and 3/214 (1.4%) respectively (Figure 1).

![Figure 1: Prevalence of *pfcr* K76T mutations in symptomatic documented fever, symptomatic reported fever and asymptomatic malaria](image)

Wild type / Mutant (Mixed) genotypes were significantly higher in symptomatic malaria infected children compared to asymptomatic malaria infected children (using generalized estimating equations models p<0.05). No pure wild type was observed.

The prevalence of *pfmdr*-1 N86Y allele wild type, mutant and mixed infection in symptomatic (documented fever), symptomatic (reported fever) and asymptomatic malaria were: 115/243 (47.3%), 40/243 (16.4%) and 88/243 (36.2%): 67/208 (32.2%), 22/208 (10.6%) and 119/208 (57.2%); 47/229 (20.5%) 64/229 (28.0%) and 118/229 (51.5%) respectively (figure 2):
Figure 2: Prevalence of pfmdr1 N86Y mutations in symptomatic documented, symptomatic reported and asymptomatic malaria

Wild type genotypes were significantly higher in symptomatic malaria infected children compared to asymptomatic malaria infected children using generalized estimating equations models (p<0.05).

The prevalence of pfmdr1 D1246Y allele of wild type, mutant and mixed infection in symptomatic (documented fever), symptomatic (reported fever) and asymptomatic malaria were: 24/207 (11.6%), 72/207 (34.8%), 111/207 (53.6%); 8/206 (3.9%), 90/206 (43.7%) and 108/206 (52.4%); 7/231 (3%) 86/231 (37.3%) 138/231 (59.7%) respectively (figure 3).
**Figure 3:** Prevalence of *pfmdr1* D1246Y mutations in symptomatic documented, symptomatic reported and asymptomatic malaria.

Wild type genotypes were significantly higher in symptomatic malaria infected children compared to asymptomatic malaria infected children using generalized estimating equations models (p<0.05).

### 4.3 Association of transporter polymorphisms mediating resistance to antimalarial drugs and complexity of infection with clinical presentations

Generalized Estimating Equations (GEE) models were fitted for each of the three genes (K76T, N86Y and D1246Y) separately accounting for clustering. Odds ratios plus 95% confidence intervals based on robust standard errors were reported together with the corresponding P values. Unadjusted analysis was done to show the association between each of the factors (*pfcrt* K76T, *pfmdr1* N86Y and *pfmdr1* D1246Y) with the response (documented fever versus asymptomatic). Multivariate analysis (adjusted) was later done adjusting for age, mean complexity of infection and parasite density (Table 4).
Table 4: Risk factors associated with symptomatic or asymptomatic malaria

<table>
<thead>
<tr>
<th>Factor</th>
<th>Alleles</th>
<th>Unadjusted</th>
<th>P value</th>
<th>Adjusted</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>K76T</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutant</td>
<td>1</td>
<td>6.2 (2.0 – 19.5)</td>
<td>0.002</td>
<td>4.3 (1.2 – 16.3)</td>
<td>0.029</td>
</tr>
<tr>
<td>Mixed</td>
<td></td>
<td>0.5 (0.3 – 0.7)</td>
<td>&lt;0.001</td>
<td>0.6 (0.3 – 1.0)</td>
<td>0.048</td>
</tr>
<tr>
<td>Age group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 5 years</td>
<td>1</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>5 – 15 years</td>
<td></td>
<td>0.5 (0.3 – 0.7)</td>
<td>&lt;0.001</td>
<td>0.6 (0.3 – 1.0)</td>
<td>0.076</td>
</tr>
<tr>
<td>N86Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>1</td>
<td>0.3 (0.1 – 0.5)</td>
<td>&lt;0.001</td>
<td>0.3 (0.1 – 0.6)</td>
<td>0.001</td>
</tr>
<tr>
<td>Mutant</td>
<td></td>
<td>0.5 (0.3 – 0.7)</td>
<td>&lt;0.001</td>
<td>0.6 (0.3 – 1.0)</td>
<td>0.076</td>
</tr>
<tr>
<td>Mixed</td>
<td></td>
<td>0.3 (0.2 – 0.5)</td>
<td>&lt;0.001</td>
<td>0.3 (0.2 – 0.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 5 years</td>
<td>1</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>5 – 15 years</td>
<td></td>
<td>0.5 (0.3 – 0.7)</td>
<td>&lt;0.001</td>
<td>0.6 (0.3 – 1.0)</td>
<td>0.076</td>
</tr>
<tr>
<td>D1246Y</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>1</td>
<td>0.2 (0.1 – 0.6)</td>
<td>0.001</td>
<td>0.2 (0.1 – 0.8)</td>
<td>0.018</td>
</tr>
<tr>
<td>Mutant</td>
<td></td>
<td>0.5 (0.3 – 0.7)</td>
<td>&lt;0.001</td>
<td>0.6 (0.3 – 1.1)</td>
<td>0.082</td>
</tr>
<tr>
<td>Mixed</td>
<td></td>
<td>0.2 (0.1 – 0.6)</td>
<td>0.001</td>
<td>0.2 (0.1 – 0.7)</td>
<td>0.010</td>
</tr>
<tr>
<td>Age group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 5 years</td>
<td>1</td>
<td></td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>5 – 15 years</td>
<td></td>
<td>0.5 (0.3 – 0.7)</td>
<td>&lt;0.001</td>
<td>0.6 (0.3 – 1.1)</td>
<td>0.082</td>
</tr>
</tbody>
</table>

OR – Odds ratio, K76T, N86Y and D1246Y are plasmodium falciparum transporter polymorphisms at codons 76, 86 and 1246 of Pfcrt and Pfmdr1 respectively.

For Pfmdr1 N86Y and D1246Y polymorphisms, the prevalence of wild type (WT) genotypes was significantly higher in febrile compared to asymptomatic children, Pfmdr1 N86Y WT versus mutant: OR 4.1 (2.0-8.3), p<0.001; Pfmdr1 D1246Y Wild type versus mutant: OR 4.9 (1.5-15.8), p=0.008. For pfcrt K76T polymorphisms, there was no pure wild type. Mixed infections were significantly higher in symptomatic compared to asymptomatic infections, pfcrt K76T mixed versus mutant: OR 4.4 (95% CI 1.3-15.1), p=0.02; Comparisons between febrile children and those with undocumented fever produced intermediate results.

4.4 Complexity of plasmodium falciparum infections in symptomatic documented fever, symptomatic reported fever and asymptomatic malaria

The mean complexity of infection was high in children with symptomatic (3.3) compared to those with asymptomatic malaria (2.6). The number of alleles varied in children with
symptomatic and those with asymptomatic malaria for 3D7. Each band represented an allele in an individual as shown in Figure 4:

![Figure 4: Strains in the 3D7 allelic family of msp-2 gene on a 2.5% agarose gel.](image)

Samples on Lanes 1, 2 and 3 had three alleles each, lanes 4, 5, 6 and 7 had one allele each, lanes 8 and 9 had two alleles each as shown on the gel above.

Controls included no DNA (Neg) and DNA from laboratory strains (3D7 and HB3). Sizes were compared based on 50 bp markers (M). 1, 2,3,4,5,6,7,8 and 9 were samples. Each band represented a particular strain in the 3D7 allelic family of msp-2 gene.

Each band represented a particular strain in the FC27 allelic family of MSP-2 gene as shown in the figure 5:
Sizes were compared based on 50 bp markers (M). 1, 2, 3, 4, 5, 6, 7, 8 and 9 were samples. Each band represented a particular strain in the FC27 allelic family of msp-2 gene.

The mean infection complexity for symptomatic and asymptomatic malaria infected children was 3.26 and 2.56 respectively. The range, median and mean is as summarized in the Table below:

<table>
<thead>
<tr>
<th>SYMPTOMATIC MALARIA</th>
<th>ASYMPOTOMATIC MALARIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of samples</td>
<td>214</td>
</tr>
<tr>
<td>Minimum</td>
<td>1</td>
</tr>
<tr>
<td>25% Percentile</td>
<td>2</td>
</tr>
<tr>
<td>Median</td>
<td>3</td>
</tr>
<tr>
<td>75% Percentile</td>
<td>5</td>
</tr>
<tr>
<td>Maximum</td>
<td>7</td>
</tr>
<tr>
<td>Mean</td>
<td>3.257</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>1.68</td>
</tr>
<tr>
<td>Std. Error</td>
<td>0.1148</td>
</tr>
<tr>
<td>Lower 95% CI of mean</td>
<td>3.031</td>
</tr>
<tr>
<td>Upper 95% CI of mean</td>
<td>3.483</td>
</tr>
<tr>
<td>Sum</td>
<td>697</td>
</tr>
<tr>
<td>Number of samples</td>
<td>201</td>
</tr>
<tr>
<td>Minimum</td>
<td>1</td>
</tr>
<tr>
<td>25% Percentile</td>
<td>1</td>
</tr>
<tr>
<td>Median</td>
<td>2</td>
</tr>
<tr>
<td>75% Percentile</td>
<td>3</td>
</tr>
<tr>
<td>Maximum</td>
<td>9</td>
</tr>
<tr>
<td>Mean</td>
<td>2.562</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>1.367</td>
</tr>
<tr>
<td>Std. Error</td>
<td>0.09639</td>
</tr>
<tr>
<td>Lower 95% CI of mean</td>
<td>2.372</td>
</tr>
<tr>
<td>Upper 95% CI of mean</td>
<td>2.752</td>
</tr>
<tr>
<td>Sum</td>
<td>515</td>
</tr>
</tbody>
</table>

Higher infection complexity was associated with symptomatic malaria and low infection complexity was associated with asymptomatic malaria (p<0.05) using paired t-test.

Figure 5: Strains in the FC27 allelic family of msp-2 gene were amplified, and products were resolved by 2.5% agarose gel electrophoresis.

Samples on lanes 1 and 3 had two alleles each; samples on lanes 2, 4, 5, 6, 7, 8 and 9 had one allele as shown in the gel above. Controls included no DNA (Neg) and DNA from laboratory strains (3D7 and HB3).
CHAPTER FIVE

DISCUSSION

5.1 Discussion

In this study, differences in polymorphism prevalence and complexity of infection (COI) in *P. falciparum* parasites associated with different clinical presentations (documented fever, reported fever and asymptomatic malaria) in a highly malaria endemic area in Uganda were explored. This was done by analyzing single nucleotide polymorphisms in *pfcrt* and *pfmdr 1*, genes encoding putative drug transporters particularly: *pfcrt* K76T, *pfmdr 1* N86Y, and *pfmdr 1* D1246Y mutations using Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP). Data for complexity of infection was generated by genotyping for *plasmodium falciparum 3D7* and FC27 alleles of the highly polymorphic merozoite surface protein-2 (msp-2) gene.

From this study, no samples had a pure wild type genotype for *pfcrt* K76T polymorphisms. Mixed (Wild type/Mutant) genotypes were more common in children with documented fever than in the other clinical categories for *pfcrt* K76T polymorphism. For both *pfmdr 1* N86Y and D1246Y polymorphisms, the prevalence of wild type genotypes was greater in children with documented fever than in children in the other clinical categories.

The mean complexity of infection was greater in children with documented fever than in the other clinical categories. Furthermore, complexity of infection was similar in children with reported, but undocumented fever and asymptomatic malaria suggesting that, as suspected, most children with undocumented fever were not actually ill with malaria.
Our principal comparison was between children with fever and those who were asymptomatic since, as above, children with reported, but undocumented fever had uncertain clinical status.

In highly malaria endemic areas, risks of malaria vary with age, as immunity develops gradually in children. In addition, children were enrolled from 20 geographical clusters, which might differ in host or parasite factors. Therefore, a multivariate analysis adjusting for age, COI, and parasite density was conducted, accounting for clustering with generalized estimating equation models fitted for each polymorphism, with robust standard errors. With both unadjusted and adjusted multivariate analysis, for all 3 polymorphisms, the prevalence of wild type (or mixed for \textit{pfcrt} K76T) genotypes was significantly higher in children with documented fevers compared to asymptomatic children. In the adjusted analysis, odds ratios for genotypes in children with documented fever were 4.4 (95% CI 1.3-15.1, \(p=0.02\)) for \textit{pfcrt} K76T mixed versus mutant; 4.1 (2.0-8.3, \(p<0.001\)) for \textit{pfmdr 1} N86Y wild type versus mutant; and 4.9 (1.5-15.8, \(p=0.008\)) for \textit{pfmdr 1} D1246Y wild type versus mutant. With the adjusted multivariate analysis the mean COI and geometric mean parasite density were both significantly higher in children with documented fever compared to those who were asymptomatic.

These results indicate that, in a cross-sectional survey of children in a region of very high malaria transmission intensity, those harboring \textit{P. falciparum} with wild type genotypes were more likely to be febrile than those with parasites containing mutations associated with resistance to chloroquine and amodiaquine. These results suggest that wild type parasites are more capable of causing clinical illness than those with resistance-mediating mutations in \textit{pfcrt} and \textit{pfmdr 1}. Thus, resistance-mediating polymorphisms may come with some cost in decreased parasite virulence. Similar results were seen in a recent study from Benin (Ogouyemi-Hounto \textit{et al.}, 2013). In Benin the prevalence of the \textit{pfcrt} 76T mutation was very high, and unchanged
between symptomatic and asymptomatic children. However, the \textit{pfmdr 1} 86Y mutation was more prevalent in asymptomatic, compared to symptomatic children, consistent with results from this current study. The \textit{pfmdr 1} 1246Y mutation is uncommon in West Africa, and was not studied.

Findings from this current study on transporter polymorphisms differ from those of the study that was conducted in western Kenya (Zhong et al., 2008) in which it was found out that the prevalence of transporter polymorphisms were not significantly different in symptomatic and asymptomatic malaria infected children. However, in that study, parasites from low land and high land areas with different transmission intensities were analyzed. In addition, the age group (6 – 14 years) was different from that of the current study. Of interest, parasites harboring the \textit{pfcr7} 76T, \textit{pfmdr 1} 86Y, and \textit{pfmdr 1} 1246Y mutations have decreased sensitivity to chloroquine and amodiaquine, but increased sensitivity to other important antimalarials, including artemisinins, lumefantrine, and mefloquine. Thus, the wild type parasites that we found to be more likely to cause symptomatic malaria are less susceptible to key drugs than mutant parasites. In other words, the polymorphisms that appear to increase the virulence of malaria parasites also increase the likelihood that parasites will be inadequately treated with key antimalarials, including both components of the Ugandan national regimen, artemether/lumefantrine.

These results are of a concern, as they suggest that the same parasite polymorphisms that favor survival after treatment with artemether/lumefantaine also favor progression of infection to clinical illness. However, it is important to note that the studied \textit{pfmdr 1} polymorphisms have only modest effects on drug sensitivity, and in fact recent trials have shown continued excellent antimalarial efficacy for artemether/lumefantrine in Uganda (Yeka et al., 2013).
An important limitation of this study is that, due to its cross-sectional design, reliable information on prior treatment histories of study subjects was not available. Prior treatment with artemether/lumefantrine leads to increased prevalence of \textit{pfmdr 1} wild type genotypes in parasites causing subsequent infections within two months after treatment (Baliraine & Rosenthal, 2011). Although it seems unlikely, we cannot exclude the possibility that symptomatic parasitemic children were more likely than asymptomatic parasitemic children to have had recent prior episodes of malaria that were treated with artemether/lumefantrine, and thus that prior treatment rather than inherent differences in parasite virulence explained the association between wild type genotype and symptomatic malaria. Nonetheless, potential increased virulence for parasites with decreased drug sensitivity is of concern.
CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The following conclusions were made from this study:

1. Wild type genotypes for pfmdr 1 N86Y and D1246Y are associated with symptomatic falciparum malaria.
2. Mixed (Wild type/Mutant) genotypes for pfcr K76T gene are associated with symptomatic falciparum malaria.
3. Parasite changes selected by leading artemether combination therapy may increase the virulence of malaria parasites.

6.2 Recommendations

The following recommendations were made from this study:

1. An additional study is needed in other populations to determine if the identified relationships will be seen in populations with lower malaria transmission intensity.
2. To rule out the influence of prior malaria therapy on parasite genotypes, children who have had no treatment with artemether lumefantrine in the last 60 days should be studied.
REFERENCES


Tran, C. V., & Saier, M. H., Jr. (2004). The principal chloroquine resistance protein of *Plasmodium falciparum* is a member of the drug/metabolite transporter superfamily. [In Vitro]. *Microbiology, 150*(Pt 1), 1-3.


APPENDICES

APPENDIX 1: CROSS-SECTIONAL SURVEY

Research participant assent form for children

Protocol Title: ACT PRIME Study: Evaluating the impact of enhanced health facility-based care for malaria and febrile illnesses in children

Site of Research: Tororo, Uganda

Principal Investigators: Dr. Sarah Staedke

Date: 18 September 2010

—I am being asked to decide if I want to be in this research study.
—I know that I will have to see the survey field workers today.
—the field workers will talk to me, ask me questions, and examine me.
—I know I will have a few drops of blood drawn from my finger today.
—I asked and got answers to my questions. I know that I can ask questions about this survey at any time.
—I know that I can stop being in this survey at anytime without anyone being mad at me.

Mark one box with X:

I DO CONSENT: ☐ I hereby agree to take part in this survey

I DO NOT CONSENT: ☐ I do not wish to take part in this survey
Name of child:

Signature or fingerprint of child:   Date:

**Witness**: I hereby confirm that the study has been explained to the child. All questions (if any) have also been answered to his/her satisfaction, and he/she has, of his own free will, consented to take part in the survey.

<table>
<thead>
<tr>
<th>Name of witness:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signature of witness:</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name of person explaining study:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signature:</td>
</tr>
</tbody>
</table>
INTRODUCTION

While your child is in this study, there may be blood samples taken from them that may be useful for future research. These samples will be stored long-term at Makerere University Medical School and the London School of Hygiene and Tropical Medicine, and the University of California, San Francisco. Samples may also be shared with investigators at other institutions.

WHAT SAMPLES WILL BE USED FOR

Your child’s blood and the malaria parasites in it will be used to study malaria and the response of this disease to treatment. Results of these studies will not affect your child's care.

1. These samples will be used for future research to learn more about malaria and other diseases.
2. Your child’s samples will be used only for research and will not be sold or used for the production of commercial products.

3. Genetic research may be performed on samples. However, no genetic information obtained from this research will be placed in your child’s medical records. These samples will be identified only by codes so that they cannot be readily identified with your child.

LEVEL OF IDENTIFICATION

Your child’s samples will be coded so that your child’s name cannot be readily identified. Reports about research done with your child’s samples will not be put in their medical record and will be kept confidential to the best of our ability. In the future, researchers studying your child’s samples may need to know more about your child, such as their age, gender, and race. If this information is already available because of your child’s participation in a study, it may be provided to the researcher. Your child’s name or anything that might identify them personally will not be provided. You will not be asked to provide additional consent.

RISKS

There are few risks to your child from future use of their samples. A potential risk might be the release of information from your child’s health or study records. Reports about research done with your child’s samples will not be put in their health record, but will be kept with the study records. The study records will be kept confidential as far as possible.

BENEFITS

There will be no direct benefit to your child. From studying your child’s samples we may learn more about malaria or other diseases: how to prevent them, how to treat them, how to cure them.
RESEARCH RESULTS/MEDICAL RECORDS

1. Results from future research using your child’s samples may be presented in publications and meetings but patient names will not be identified.

2. Reports from future research done with your child’s samples will not be given to you or your child’s doctor. These reports will not be put in your child’s medical record.

QUESTIONS

If you have any questions, comments or concerns about the future use of your child’s specimen’s, first talk to the researchers. You may also Contact Dr. Sarah Staedke or other members of the Uganda Malaria Surveillance Project on telephone number 0414-530692. If for any reason you do not wish to do this, or you still have concerns about the future use of your child’s specimens, you may contact Dr Charles Ibingira, Makerere University Faculty of Medicine Research and Ethical Committee at telephone number 0414-530020.

FREEDOM TO REFUSE

You can change your mind at any time about allowing your child’s samples to be used for future research. If you do, contact Dr. Staedke or other members of the Uganda Malaria Surveillance Project at the numbers listed above. Then your child’s samples will no longer be made available for research and will be destroyed. Whether or not you allow us to use your child’s samples in future research will not have any effect on your child’s participation in this study or future participation in other studies.

WHAT YOUR SIGNATURE OR THUMBPRINT MEANS

Your signature or thumbprint below means that you understand the information given to you in this consent form about your child’s specimens to be used for future research. If you wish to
allow your child’s specimens to be used for future research, you should sign or place your thumbprint below.

**WE WILL GIVE YOU A COPY OF THIS SIGNED AND DATED CONSENT FORM**

<table>
<thead>
<tr>
<th>Name of Participant (printed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name of Parent/Guardian</td>
</tr>
<tr>
<td>Signature or Fingerprint * of Parent/Guardian Date/Time</td>
</tr>
<tr>
<td>Name of Investigator Administering Consent (printed) Position/Title</td>
</tr>
<tr>
<td>Signature of Investigator Administering Consent Date/Time</td>
</tr>
</tbody>
</table>

*If the parent or guardian is unable to read and/or write, an impartial witness should be present during the informed consent discussion. After the written informed consent form is read and explained to the participant and parent or guardian, and after they have orally consented to their child’s participation in the trial, and have either signed the consent form or provided their
fingerprint, the witness should sign and personally date the consent form. By signing the consent
form, the witness attests that the information in the consent form and any other written
information was accurately explained to, and apparently understood by the parent or guardian,
and that informed consent was freely given by the patient and parent or guardian.

__________________________________________
Name of Person Witnessing Consent (printed)

__________________________________________
Signature of Person Witnessing Consent                                      Date/Time