ANTIMALARIAL SUSCEPTIBILITY OF *Plasmodium falciparum* ISOLATES FROM PATIENTS PRESENTING WITH UNCOMPLICATED MALARIA AT MULAGO HOSPITAL

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A DISSERTATION SUBMITTED TO MAKERERE UNIVERSITY IN PARTIAL- FULFILLMENT FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN PHARMACOLOGY-OF MAKERERE UNIVERSITY.

OCTOBER 2007
DECLARATION

I, Nakaziba Rebecca, certify that the work in this book was designed, done and presented by me. Except where acknowledged, the views expressed in the text are mine. This work has never been presented to any institution or any forum for the purpose of getting an academic award.

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ACKNOWLEDGEMENT

Great thanks to the great Lord and God Almighty for being ever present in thick and thin; for guarding my heart from crumbling during tough moments.

I thank my lovely Dad Mr. Mufumba George William for the big heart; for not giving me up when he could have; for his counsel and support since my childhood. God bless him. I thank my mother, Mrs. Mufumba Florence, for her tender love and care. May God reward her.

I greatly thank my supervisors, Dr. Paul Waako and Prof. Jasper Ogwal - Okeng for their being ever ready to help when needed and for the great counsel, guidance, support and encouragement. I will live to remember them. God bless them.

Thanks to the vicres project of the Inter-University Council for East Africa for funding this project.

In a special way, I thank the Head, Department of Pharmacology and Therapeutics, Dr. Paul Waako, for always being willing to help where need be and for his fatherly love and care. May God reward him.

I thank Mr. Niale Mohamed for donating to me the antimalarial drugs used for this study and the support and encouragement. God bless him for the big heart.

I also thank JCRC- Immunology laboratory for helping me with the ELISA reader.

Thanks to Ms Katura Esther for sharing information on methods with me and Mr. Sebisubi Fred, for his counsel and readiness to assist.

Finally, I thank the rest of the Departmental staff members and colleagues for all assistance rendered to me during the course of time. God bless them.
DEDICATION

To my dear parents, Mr. and Mrs. Muñumba George William and to my little brother

Brian Joshua Mufumba.
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<td>CRPF:</td>
<td>Chloroquine Resistant <em>Plasmodium falciparum</em></td>
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<td>HIV:</td>
<td>Human Immunodeficiency Virus</td>
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<td>WHO:</td>
<td>World Health Organization</td>
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<td>OPD:</td>
<td>Out Patient Department</td>
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<td>AIDS:</td>
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<td>DHFR:</td>
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<td>PCR:</td>
<td>Polymerase Chain Reaction</td>
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<td>DNA:</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>IC₅₀:</td>
<td>50% Inhibitory Concentration</td>
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<td>SP:</td>
<td>Sulfadoxine-Pyrimethamine</td>
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<td>EDTA:</td>
<td>Ethylene Diamine Tetra Acetic acid</td>
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<td>APAD:</td>
<td>3-Acetyl pyridine adenine dinucleotide</td>
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<tr>
<td>AMDP:</td>
<td>Antimalarial Drug Policy</td>
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<td>RBM:</td>
<td>Roll Back Malaria</td>
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ABSTRACT

Introduction and objectives

Malaria remains the greatest cause of morbidity and mortality in sub-Saharan Africa.

This work was done to determine the sensitivity of *Plasmodium falciparum* isolates to the currently used antimalarial drugs (Chloroquine diphosphate, Artemether, Quinine sulfate, Amodiaquine, Lumefantrine), and drug combinations (Artemether-Lumefantrine, Artemether-Amodiaquine, Artemether –Quinine sulfate) in Uganda, particularly in Mulago-Hospital.

Method

Wild strains of *P. falciparum* obtained from patients attending the out patient department of Mulago Hospital, Kampala, Uganda, were screened for sensitivity to the above antimalarial drugs using the parasite lactate-dehydrogenase assay described by Makler et al, 1993 in which parasite growth and thus density is determined by the amount of the enzyme (which reduces APAD causing it to turn blue with the NBT dye) produced by the parasites. The parasite sensitivity to the drugs was described by the IC<sub>50</sub> i.e. the drug concentration added to an *in vitro* culture of parasites that reduces parasite growth by 50%.

Results

Most of the test samples were sensitive to Quinine sulfate among the single drugs followed by Artemether, the least being Lumefantrine. Artemether – Lumefantrine was the most active among the combined drugs and Artemether – Quinine was the least active.

Conclusion

Quinine was the most active single drug and Artemether – Lumefantrine was the most active among the combined drugs.
CHAPTER ONE
INTRODUCTION

1.1 Background

Malaria remains one of the greatest causes of morbidity and mortality in the tropical and sub-tropical parts of the world. It is estimated that about 300-500 million clinical cases of malaria occur annually and that about 1.5-2.7 million people die from the disease each year (WHO, 1996). The burden of *falciparum* malaria is on the increase and as has probably always been the case, it is carried mainly by tropical Africa (Peter *et al.*, 2004).

*P. falciparum* is the most virulent parasite strain responsible for the high morbidity and mortality associated with the disease. The problem is compounded by the spread of drug resistant strains of the parasite. Chemotherapy is the most important approach to malaria control and is likely to remain so for a considerable time. Unfortunately, the extensive development of resistance to currently used drugs calls for the development of novel antimalarials (Peter *et al.*, 2004).

In Uganda, 95% of the country is endemic to malaria, the disease accounting for 25-40% of out-patient consultations, 20% of in-patient admissions and 9-14% of in-patient deaths, most of whom are children below 5 years of age (Ministry of Health, 2005). Little information is known about the sensitivity of *P. falciparum* strains to the currently used antimalarial drugs in Uganda. Most malaria treatment out-come data are based on field studies which are very expensive and require a lot of time to conduct. It is therefore
necessary to find out the \textit{in vitro} susceptibility of \textit{P. falciparum} to the drugs in use in order to be able to administer effective drugs.

This study determined the \textit{in vitro} sensitivity of \textit{P. falciparum} obtained from a group of patients attending the OPD of Mulago hospital to currently used antimalarials singly and in combinations, (chloroquine, artemether, quinine, amodiaquine, lumefantrine, and the combinations: artemether - lumefantrine, artemether-amodiaquine and artemether-quinine), in Uganda.

1.2 Problem Statement

Improper use of drugs and poor drug quality are among the causes of increased \textit{P. falciparum} resistance to antimalarial drugs in Uganda. This has resulted into poor treatment outcomes, which has led to increased morbidity and mortality, high cost of treatment, and prolonged stay of patients in health units. The increased patient load has led to reduced consultation time, poor quality of life and so many other devastating effects. In Uganda, available data on the sensitivity profile of the malaria parasite are based on \textit{in vivo} studies that take long, are very expensive, and cannot cover a large spectrum of the drugs in use. Therefore, it was necessary to establish the \textit{in vitro} susceptibility of the \textit{P. falciparum} in Uganda to the antimalarials in use to provide baseline information on the sensitivity status of the parasite and in order to be able to administer drugs which are effective.
1.3 Objectives of the study

1.3.1 General objective

The aim of this study was to find out the antimalarial sensitivity of *P. falciparum* strains isolated from patients attending Mulago Hospital OPD with uncomplicated malaria to commonly used antimalarial drugs and drug combinations.

1.3.2 Specific objectives

This study had the following specific objectives:

1. To find out the sensitivity of *P. falciparum* to commonly used single drugs in an *in vitro* system.

2. To establish the sensitivity of *P. falciparum* to drug combinations in an *in vitro* system.

1.4 Significance of the study

The determination of the sensitivity pattern of *P. falciparum* to the single drugs and drug combinations will help to identify the drug or drug combination to which the *P. falciparum* parasite is most sensitive. This information will guide health care providers on the drugs/drug combinations, which are more effective and will result into improved treatment outcome, and better quality of life.
1.5 Study Justification

Malaria is a leading cause of mortality in Uganda. The malaria treatment policy has been changed two times in the past 10 years due to the changing drug resistance patterns in the country. This raises the need for a high output system to provide information on the resistance patterns of the malaria parasite. This information will help to formulate guidelines for the treatment policy in health facilities.

1.6 Research Question

1. What is the sensitivity of the strains of *P. falciparum* isolated from patients who present with uncomplicated malaria in Mulago Hospital to currently used antimalarial drugs and drug combinations, (chloroquine diphosphate, amodiaquine, artemether, lumefantrine, quinine sulfate, and to the combinations: artemether-lumefantrine, artemether-amodiaquine, artemether–quinine sulfate)?
CHAPTER TWO

LITERATURE REVIEW

2.1 The Global burden of malaria

Malaria is mosquito-borne and one of the major killer disease in the tropical and subtropical regions of the world. According to the World Health Organisation, malaria is a significant public health problem in more than 90 countries inhabited by some 2400 million people (about 40% of the world’s population) (Rang et al., 2003). There are an estimated 300-500 million clinical cases each year with more than 90% of these occurring in the sub-Saharan Africa causing up to 2.7 million deaths per year, the vast majority of which is among young children in Africa, especially in remote rural areas with limited or no access to medical care. In some parts of Africa, malaria kills 3000 children under 5 years of age each day, a death toll much greater than that associated with HIV/AIDS (Rang et al., 2003). Other high-risk groups include women during pregnancy, refugees and labourers entering endemic regions. In countries where the disease is rife, malaria imposes a huge economic burden (Rang et al., 2003). Malaria is the major cause of morbidity and mortality in most tropical countries (WHO, 1996). About 90% of all malaria deaths in the world today occur in Africa, south of the Sahara. This is because the majority of infections in Africa are caused by *P. falciparum*, the most dangerous of the four human malaria parasites. Also the distribution of the malaria vector, the mosquito *Anopheles gambiae*, is densely spread in Africa and is the most difficult to control. Malaria affects the lives of almost all people living in Africa especially in areas of stable transmission. In these areas, very young children and pregnant women are the population groups at highest risk for
malaria morbidity and mortality. In Africa, 90% of all deaths occur in young children (WHO, 2003).

In Uganda, malaria currently accounts for: 25-40% of all outpatient visits at health facilities, 20% of hospital admissions, 9-14% of in-patient deaths, a case fatality rate of 3-5% (under estimate), 23.4% of total discounted life years lost, 23% and 11% of deaths among the under 5s in high and medium malaria transmission areas respectively, and is a major killer of refugees and internally displaced people. Apart from malaria causing ill-health and death, it also has a great impact on the economic development of individuals, families, the community and the nation at large by: direct costs in the form of for example, treatment, seeking treatment, and funeral expenses. Malaria is also a leading cause of poverty in Uganda (Ministry Of Health, 2005).

2.2 Causative organism and its life cycle

Malaria is caused by protozoa of the genus *plasmodium* present in the human host either in the erythrocytic (blood) stage or the exoerythrocytic (tissue) stage. Four species of *plasmodium* are responsible for human malaria: *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium falciparum*. Although all may cause severe illness, *P. falciparum* causes most of the serious complications and deaths. The mosquito becomes infected by taking human blood that contains parasites in the sexual form. The sporozoites that develop in the mosquito are then inoculated into humans at its next feeding. In the first stage of development in humans (the exoerythrocytic stage), the sporozoites multiply in the liver to form tissue schizonts. Later, the parasites escape from the liver into the blood stream as merozoites to initiate the erythrocytic stage. In this stage, they invade red blood
cells, multiply in them to form blood schizonts, and finally rapture the cells, releasing a new crop of merozoites. This cycle may be repeated many times. Meanwhile, the gametocytes form and are released into the circulation, where they may be taken in by another mosquito (Bertram, 1998).

2.3 Methods of controlling malaria

Currently measures for malaria control include chemotherapy, chemoprophylaxis, preventing vector contact with people by the use of bed nets, mosquito repellants and vector control such as the use of DDT, clearing of bushes from around households, etc. The primary approach to malaria control in sub-Saharan Africa has been chemotherapy. However the provision of effective chemotherapy, which had been the mainstay of malaria control in countries such as Uganda, is now hampered by the emergence and spread of chloroquine resistant *Plasmodium falciparum* (Nuwaha, 2001, Bloland *et al.*, 1998)

2.4 Malaria chemotherapy

The effectiveness of antimalarial agents varies depending on the parasite species and the stages in their life cycles. Proper use of antimalarial drugs is based on knowledge of their effects on the parasite at various stages of the life cycle. Drug treatment chiefly involves the use of aminoquinolines, such as chloroquine, its analogs and a number of additional drugs such as sulfadoxine and pyrimethamine, for the erythrocytic stage and chiefly primaquine for the exoerythrocytic stage. Newer compounds such as mefloquine, artemisinin (also known as ginghaosu) and halofantrine are also used, and several
antibiotics are often administered in combination with antimalarial agents. Older agents that have wide use include quinine and quinidine (Lemuel et al., 1991).

Different classes of drugs are used to treat malaria. Examples include:

- 4-aminoquinolones, such as chloroquine and amodiaquine, which have a marked and rapid schizonticidal activity; antifolate drugs, for example sulfadoxine and pyrimethamine, which act against parasite specific enzymes, dihydropteroate synthetase and dihydrofolate reductase, and are highly active blood schizonticides against *P. falciparum*; and proguanil, a synthetic biguanide derivative of pyrimidine with marked effect on the tissue stages of *P. falciparum*, *P. vivax* and *P. ovale* and is a dihydrofolate reductase inhibitor acting through its major metabolite cycloguanil. Proguanil can be used in combination with chloroquine.

Other classes of drugs for malaria are: 8-aminoquinolones such as primaquine which is highly active against the gametocytes of all malaria parasite species found in humans, and against hypnozoites of the relapsing malaria parasites *P. vivax* and *P. ovale*;

- quinine, quinidine and related alkaloids, which are normally effective against falciparum parasites that are resistant to chloroquine and sulfa drug- pyrimethamine combinations;
- artemisinin and its derivatives such as artesunate, arteether, and dihydroartemisinin, which are potent blood schizonticides; and antibiotics such as doxycycline, tetracycline, clindamycin and azithromycin, which are used as adjuvant drugs in combination with other antimalarial agents.
2.5 The problem of resistance

Antimalarial drug resistance is the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended, but within the limits of tolerance of the subject (WHO, 1973).

Resistance to antimalarial drugs arises as a result of spontaneously occurring mutations that affect the structure and activity at the molecular level of the drug target (Peters, 1987). Mutant parasites are selected if antimalarial drug concentrations are sufficient to inhibit multiplication of susceptible parasites but are inadequate to inhibit mutants (White, 1998; Peters, 1990).

Various factors relating to drug, parasite, and human host interactions contribute to the development and spread of drug resistance. The molecular mechanism of drug action is a critical element in the speed at which resistance develops. Drugs with a long terminal elimination half-life enhance the development of resistance particularly in areas of high transmission. Similarly, increased drug pressure is a significant contributor to drug resistance (Watkins and Mosobo, 1993). Parasite factors associated with resistance include the Plasmodium species concerned and the intensity of transmission. Human host factors include the wide spread and/or irrational use of antimalarial drugs and the level of host immunity which acts synergistically with chemotherapy to enhance therapeutic effects and even parasite clearance of drug resistant infections (WHO, 2000).
In *P. falciparum* chloroquine resistance, the mutations occur in a transporter-like gene on the surface of the parasite food vacuole (Su X *et al*., 1997). *P. falciparum* resistance to sulfadoxine and pyrimethamine is primarily conferred by successive single point mutations in the parasite *dhfr*, the gene that encodes the target enzyme dihydrofolate reductase (DHFR), and by additional mutations in *dhps*, which encodes for the enzyme dihydropteroate synthetase (DHPS) (Triglia *et al*., 1997).

The world malaria situation is aggravated by the fact that an increased prevalence of drug-resistant strains of *P. falciparum* continue to reduce the effectiveness of most known antimalarials (White, 1992). Drug-resistant populations of *P. falciparum* are selected by widespread and improper use of antimalarials; their genetically determined drug resistance is thus promoted and propagated (Wernsdorfer and Payne, 1991).

In 1995, Nevill *et al* determined the sensitivity of *P. falciparum* isolates in Uganda to chloroquine and amodiaquine and found out that the isolates were significantly less sensitive to chloroquine than to amodiaquine.

Malaria control efforts have been greatly challenged by the emergence of *P. falciparum* resistance to chloroquine (Campbell, 1991). The spread of CQ/SP resistance poses a major threat to malaria control targets to halve the malaria burden by 2010 (WHO - RBM, 2000). Malaria incidence and mortality are increased by the continued use of ineffective drugs (Korenromp *et al*., 2003; Trape, 2001). Artemisinin-based combination therapy (ACT) is advocated for by many authorities in Africa (Attaran *et al*., 2004).
2.6 Combating antimalarials drug resistance

To try and curb resistance, malaria chemotherapy currently involves the use of combination drugs and new drugs are continually being discovered.

Since 1995, WHO and national malaria control programmes in the Africa region have responded to the spread and intensification of chloroquine resistant *P. falciparum* by strengthening national capacity in conducting 14-day in vivo drug efficacy studies (WHO, 2000). For the planning and continuous improvement of malaria control programs, the susceptibility to antimalarial drugs of the local parasite population should be monitored (Philipps *et al.*, 1998).

2.7 Drug resistance and malaria treatment policy in Uganda

The primary goal of a national Antimalarial Drug Policy (AMDP) is to minimize malaria-associated morbidity and mortality through providing affordable, safe, and effective, antimalarial drugs rationally, and limiting the development of drug resistance (Kamya *et al.*, 2002).

Drug selection pressure and the spread of resistance is promoted by unrestricted access to and inappropriate use of drugs. Therefore, the WHO/Africa Regional Office (AFRO) created guidelines in 1999 for developing, implementing and updating AMDP in Africa recommending that drug efficacy, cost, potential for cross-resistance, side-effect profile, estimated therapeutic lifespan and acceptability, be considered in the context of developing a national Antimalarial Drug Policy (Kamya *et al.*, 2002)
At the Uganda National Consensus Meeting, held in June 2000, it was recommended that, CQ should remain the first-line antimalarial in areas where clinical resistance to CQ is <25% (with SP or AQ remaining second-line therapy), but that a combination of CQ/SP should replace CQ as the first-line treatment in areas where CQ resistance is >25% (with quinine as second-line) (Kamya et al., 2002).

Regardless of baseline CQ resistance, the original policy decision was amended in November 2000, and the CQ/SP combination was recommended for use in all areas of Uganda (Kamya et al., 2002).

2.7.1 The Combination therapy strategy

The combination of antimalarial agents has been advocated for as an approach to improve therapeutic efficacy and delay drug resistance development (White, 1999). Studies suggested that CQ/SP provided more rapid resolution of symptoms and improved treatment outcome when compared with SP alone (McIntosh & Greenwood, 1998). It was however, unclear if there would be substantial benefit gained from the CQ/SP combination if resistance to either agent already existed (Kamya et al., 2002). In a trial conducted in Kampala, the addition of CQ to SP improved parasitological outcome compared with SP alone (CQ/SP vs. SP, P = 0.0026), but showed only a trend towards improved clinical outcome (P = 0.053) (Gasasira et al., 2001).

Data from three studies in Kampala, (Dorsey et al., 2001; Gasasira et al., 2001; Staedke et al., 2001), suggested that the combination of AQ/SP, both of which are affordable
antimalarials that are currently available in Uganda, was a highly effective regimen. Toxicity concerns however, has limited the enthusiasm for the widespread use of this regimen in Uganda. Although serious adverse events have been described with use of both AQ and SP, short-term treatment with both agents appears to be much safer than long-term chemoprophylaxis (Sturchler et al., 1993; Olliaro et al., 1996). Further evaluation of AQ/SP and larger studies of drug safety are needed in order to assess the risk associated with routine use of these drugs (Kamya et al., 2002).

Artemisinin compounds decrease the parasite biomass soon after administration, and thereby promote rapid clinical recovery, and decrease the chances of drug resistance development by parasites (White, 1999). The combination of AS/SP has been shown to be effective in areas with low-level resistance to SP in Africa (Von Seidlein et al., 2000). The use of artemisinin-containing combinations in Asia has been a success in the treatment of multidrug resistant malaria (Price et al., 1996; Nosten et al., 2000).

Two co-formulated antimalarial combinations, atovaquone/proguanil (Malarone) and artemether/lumefantrine (coartem), [which is the first-line antimalarial in Uganda], are currently available, but their high cost limits their use in Africa. Drug cost currently remains a hinderance to widespread use of artemisinin derivatives in Africa (Kamya et al. 2002)

In Uganda, chloroquine was replaced by chloroquine – SP (sulfadoxine-pyrimethamine) as first line treatment for uncomplicated malaria in 2002 (Kamya et al., 2002). As a result of increased resistance of Plasmodium falciparum to the drugs, artemether- lumefantrine
combination was adopted in 2004, (with artemisin - amodiaquine combination as a substitute), as first line treatment for uncomplicated malaria (Hasifa et al., 2006).

2.8 Methods for in vitro sensitivity testing of Plasmodium falciparum

The techniques for detection and quantification of malaria parasites have improved tremendously over the past three decades. Evaluation of in vitro activity of potential antimalarial agents requires an assay that is fast, reliable and easy to interpret.

2.8.1 Fluorometry: Methods based on fluorometry have been developed; they involve use of DNA staining dyes such as ethidium bromide, acridine orange and benzothiocarboxypurine. Their sensitivity parallels that of isotopic studies and geimsa-stained microscopy. Based on DNA staining, the detection of DNA fragments from dead parasites limits the specificity of the tests and it utilizes carcinogenic dyes.

2.8.2 Antigen capture: A number of techniques based on antigen capture have been developed. The key antigens include histidine-rich protein 2 surface antigen and the parasite lactate dehydrogenase enzyme. These tests are very sensitive with the latter comparable to Polymerase Chain Reaction (PCR) (Druilhe, et al., 2001). They however use monoclonal antibodies that render them expensive.

2.8.3 Isotopic assays: Isotopic assays are one of the most sensitive methods used for the detection of plasmodium. They are based on the utilisation of hypoxanthine and adenosine in nucleic acid synthesis by P. falciparum. One of these is normally used as radiolabelled
precursor. These two precursors produce virtually identical results. The isotopic assays are sensitive, reproducible and fast, but they require specific precautions for the handling of radioactive material.

2.8.4 *Parasite lactate dehydrogenase assay*: This is based on the principle that the amount of pLDH in a sample correlates with the degree of parasitemia (Makler and Hinrichs, 1993). The fact that pLDH utilizes 3-acetyl pyridine NAD (APAD) to APADH, that has the ability to reduce a yellow nitroblue tetrazolium (NBT) salt to a blue formazan product whose absorbance can be measured by microplate reader, makes it possible to measure pLDH. When used to evaluate drug sensitivity, this assay is reproducible, easy to interpret, rapid and inexpensive to perform and does not involve handling of radioactive material (Makler et al., 1993).

2.8.5 *Histidine-rich protein 2 based assays*: Which use a simple HRP2 double site sandwich ELISA to quantify parasite growth and its inhibition by detecting the HRP2 produced by *P. falciparum* during its multiplication (Noedl et al., 2003).

2.8.6 *PCR methods*: These use molecular markers to detect mutations in dhfr and dhps (Wilson et al., 2005). Other methods include the DNA hybridization assays (Waki et al., 1992).
2.9 *In vitro* tests vs. *in vivo* efficacy

*In vitro* tests are limited to research purposes for providing baseline data on drug response and to monitor cross-resistance patterns. They are a more objective method to detect drug resistance since the tests eliminate several host factors that interfere with the clear interpretation of results such as reinfections, immunity, pharmacokinetic and pharmacodynamic factors. *In vitro* assays are complementary to *in vivo* tests and their results are theoretically more directly associated with drug resistance. They are also used to describe the epidemiology of drug resistance independently of clinical studies and to screen new compounds (Ringwald and Basco, 1999).

In an experiment carried out by Ringwald and Basco (1999) to compare the simplified *in vivo* test for chloroquine resistance, the predictive value of the isotopic *in vitro* assay suggested that 86% of the patients for whom the *in vitro* test indicated the presence of chloroquine sensitive isolates actually responded adequately to the chloroquine therapy. Thus the *in vitro* assay seems to reflect well in the *in vivo* response of chloroquine treated patients evaluated by clinical and parasitological examination.
CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design

This was a cross-sectional study of the sensitivity of *P. falciparum* obtained from a group of seventeen patients presenting with uncomplicated malaria and attending the OPD of Mulago hospital.

*P. falciparum* parasites were cultured and challenged with chloroquine diphosphate, artemether, quinine sulfate, amodiaquine, lumefantrine, and to the combinations: artemether + lumefantrine (1:6), artemether- amodiaquine (1:3), artemether-quinine sulfate (1:2). The parasite response to the drugs was described by the amount of drug that inhibited parasite growth (indicated by the amount of pLDH) by 50 %.

3.2 Study setting

The study was conducted in Mulago Hospital and Makerere University Medical School, Department of Pharmacology and Therapeutics, Clinical Pharmacology Laboratory. *P. falciparum* parasites were obtained from patients presenting with uncomplicated malaria at the out patient department of Mulago Hospital.

3.3 Sourcing of malaria parasites

Wild *Plasmodium falciparum* parasite strains were obtained from blood samples from 17 symptomatic malaria patients attending the out patient department of Mulago Hospital.
3.4 Sampling

Altogether, 27 patients who presented with uncomplicated malaria at the outpatient department of Mulago Hospital were studied using purposive sampling. However, results of only 17 patients were considered. The other samples got contaminated by fungi.

3.5 Eligibility criteria

3.5.1 Inclusion criteria

Only patients with parasite counts over 1000/mm³ blood, no previous antimalarial treatment for a period of one month as reported by the patient (or care taker), informed consent, and willingness to give a venous blood sample.

3.5.2 Exclusion criteria.

Patients with complicated forms of malaria were excluded from the study. This was not because it would affect the results of the study but for convenience.

3.6 Preparation of blood sample

Venous blood samples were collected in tubes coated with the anticoagulant EDTA. The blood was then centrifuged at 1200 rpm to obtain red blood cells.
3.7 Preparation of drugs

3.7.1 Preparation of stock drug solutions

Standard drugs offered by Mr. Ntale Mohamed, (Pharmacology Laboratory, Department of Pharmacology and Therapeutics, Makerere University Medical School), were used to prepare the drug solutions.

Amodiaquine: – 0.8mg of Amodiaquine was dissolved in 2mls methanol. The mixture was shaken until a solution was formed. Lumefantrine: – 0.5mg of Lumefantrine was dissolved in 2mls methanol. The mixture was treated as above. However, Lumefantrine was only partially soluble. Quinine: – 0.7mg of Quinine sulfate was dissolved in 2mls methanol and the mixture shaken until all the drug had dissolved. Chloroquine: – 0.8mg of Chloroquine diphosphate was dissolved in 2mls methanol and then shaken until dissolution.

Artemether: – the 10μl of the intravenous (i.v) formulation of Artemether (equivalent to 0.8mg) was made up to 2mls using methanol.

All the stock solutions were stored at 4°C.

3.7.2 Preparation of working drug solutions

50μl of the stock solution for all the drugs was made to 500μl with the culture medium.

3.7.3 Preparation of drug combinations

Artemether – lumefantrine: – 2.5μl (0.1μg) of artemether plus 24μl (0.6μg) lumefantrine was made to 100μl using culture medium. Similarly, artemether – amodiaquine: - 25μl (1μg) of artemether plus 75μl (3μg) of amodiaquine was prepared.
Artemether - quinine: - 25μl (1μg) of artemether plus 57μl (2μg) of quinine was made up to 100μl with culture medium.

3.8 *In vitro* antimalarial sensitivity testing

Wild strains of *P. falciparum* from patients attending the OPD of Mulago Hospital were cultured at the Clinical Pharmacology Laboratory of the Department of Pharmacology and Therapeutics, Makerere University, Medical School.

The Nitro Blue Tetrazolium (NBT) based parasite lactate dehydrogenase (pLDH) assay, as validated by Makler *et al.*, (1993) for screening of chemotherapeutic agents, was used to determine the antiplasmodial activity of the antimalarial drugs and drug combinations.

The culture medium was made up of RPMI 1640 (Bio Whittaker) medium supplemented with Human plasma 100ml/L (obtained from the blood bank), hypoxanthine (44mg/L), N-(2-hydroxyethyl)-piperazine-N-2-ethanesulphonic acid (HEPES) (6mg/L), sodium bicarbonate (2.1g/L) and gentamycin (50mg/L). For the antiplasmodial assay, Nitro Blue Tetrazolium (NBT) (1.96mM) and phenalzine ethosulfate (PES) (0.24mM) was dissolved in de-ionized water. The Malstat reagent was made from Triton X-100 (1ml/L), 3-acetyl pyridine NAD (APAD) (0.33g/L) and TRIS buffer (3.3g/L) in de-ionized water, (all reagents were supplied by Sigma-Aldrich, South Africa).

The starting concentrations of the drugs were as follows: -

Chloroquine diphosphate (4μg), amodiaquine (4μg), quinine sulfate (3.5μg), artemether (4μg), lumefantrine (2.5μg). Artemether - lumefantrine (0.1μg: 0.6μg), artemether-amodiaquine (1μg: 3μg), and artemether – quinine sulfate (1μg: 2μg).

An ELISA reader (Cambridge Technology, Inc.) was used to determine absorbance values.
Stock solutions of the drugs were prepared and maintained in sterile conditions at 4°C for subsequent testing. Using a 96 well plate containing 100μl of culture medium, serial dilutions of the drug working solutions (100μl) were studied against suspensions of human red blood cells (100μl) with parasitaemia and haematocrit adjusted to 2%. A column of wells with non-parasitized red blood cells (100μl) at a haematocrit of 2% served as the blank while wells with parasitized erythrocytes (100μl) and no drug represented normal parasite growth. Sterile plates were placed in an airtight cabinet, gassed with a candle for about 5 minutes. The cabinet was incubated at 37°C for 48 h, exposing all parasite stages to the drug. After incubation, 15 μl of the blood suspension were transferred from each well to corresponding wells containing 30 μl of Malstat reagent on another plate. 15 μl NBT was then added to all plates and allowed to develop away from direct light. The plates were read at 650nm. The optical density of the first column (blank) was subtracted from all other readings.

3.9 Data handling and statistical analysis

The percentage of parasite survival at each concentration was determined by expressing absorbance at each concentration as a percentage of the absorbance corresponding to wells with normal parasite growth. A sigmoid curve of percentage parasite survival against logarithm of concentration generated by the prism graph pad version 3 was used to determine the IC₅₀ values.

The Nanomolar (nM) concentration of the IC₅₀ values was worked out as follows:

IC₅₀μg/100μl = [(IC₅₀ /10⁶) x (10⁶/100)] g/l; Concentration (g/l)/molecular weight (MW) = Molarity; and Molarity x10⁹ = Nanomolar.
The percentage sensitivity was obtained by counting all parasite strains with IC$_{50}$s below the sensitivity cut off value and dividing their number by the total number of parasite strains studied. Microsoft Excel was used to obtain graphs.

3.10 Quality control

The internal validity of the study was ensured by taking care of the following:

i) Blood was drawn by a qualified personnel.

ii) Laboratory procedures were carried out by a competent individual.

iii) All procedures were carried out under aseptic conditions.

iv) All these were closely monitored by me.

3.11 Ethical considerations

Patients were required to fill consent forms and have them signed. The sample form is attached as an Appendix.

Participation in the study was on a freely voluntary basis and those who decline participation were not denied the standard medical care offered for the condition.

Treatment of patients enrolled into the study conformed to the standard procedures of infection prevention.

Permission to carry out the study was obtained from:

- Department of Pharmacology and Therapeutics research committee.

- The ethics and research committee of Mulago Hospital.

- The Faculty of Medicine Research and Ethics sub-committee.

- The Uganda National Council of Science and Technology Ethics Committee.
CHAPTER FOUR

RESULTS

4.1 Study patients

Blood samples were obtained from 17 symptomatic malaria patients with parasite counts over 1000/mm³ of blood who had no previous antimalarial treatment.

4.2 Sensitivity to single drugs

Parasites from the study patients were more sensitive to quinine than all other drugs, with IC₅₀s ranging from \((0.00000 - 4.29374) \times 10^4\) nM. Parasite strains from 2 patients (6 and 7) were sensitive to all the drugs investigated. Only 3 and 4 parasite strains were absolutely resistant to chloroquine and artemether respectively. Quinine had the lowest IC₅₀ range \([(0.00000 - 4.29374) \times 10^4\) nM] followed by amodiaquine \([(0.00000 - 4.97200) \times 10^4\) nM]. Lumefantrine had the highest IC₅₀ range \([(0.00000 - 26.82420) \times 10^4\) nM]. Details of these results are presented in Tables 1 and 3 and Figure 1.

The results of antimalarial susceptibility of *P. falciparum* isolates from 17 patients who presented with uncomplicated malaria at Mulago Hospital to single drugs are presented in Table 1 below.
Table 1: The antimalarial susceptibility of *P. falciparum* isolates from 17 patients who presented with uncomplicated malaria at Mulago Hospital to single drugs.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Drug IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chloroquine</td>
<td>Artemether</td>
</tr>
<tr>
<td>1</td>
<td>0.07882</td>
<td>1.13581</td>
</tr>
<tr>
<td>2</td>
<td>0.00001</td>
<td>0.32696</td>
</tr>
<tr>
<td>3</td>
<td>***</td>
<td>5.63514</td>
</tr>
<tr>
<td>4</td>
<td>0.57016</td>
<td>***</td>
</tr>
<tr>
<td>5</td>
<td>0.84554</td>
<td>***</td>
</tr>
<tr>
<td>6</td>
<td>0.00000</td>
<td>0.00000</td>
</tr>
<tr>
<td>7</td>
<td>0.00000</td>
<td>0.00000</td>
</tr>
<tr>
<td>8</td>
<td>0.00001</td>
<td>1.46080</td>
</tr>
<tr>
<td>9</td>
<td>25.69767</td>
<td>***</td>
</tr>
<tr>
<td>10</td>
<td>***</td>
<td>14.55405</td>
</tr>
<tr>
<td>11</td>
<td>1.21841</td>
<td>0.87703</td>
</tr>
<tr>
<td>12</td>
<td>4.22286</td>
<td>***</td>
</tr>
<tr>
<td>13</td>
<td>***</td>
<td>3.09797</td>
</tr>
<tr>
<td>14</td>
<td>15.59884</td>
<td>3.89189</td>
</tr>
<tr>
<td>15</td>
<td>0.04862</td>
<td>0.00001</td>
</tr>
<tr>
<td>16</td>
<td>4.36434</td>
<td>0.22017</td>
</tr>
<tr>
<td>17</td>
<td>1.75310</td>
<td>0.65439</td>
</tr>
<tr>
<td>Cut-off IC&lt;sub&gt;50&lt;/sub&gt; (nM)</td>
<td>1.00000</td>
<td>1.50000</td>
</tr>
</tbody>
</table>

*** Refers to samples that showed absolute resistance
5.3 Sensitivity to drug combinations

The parasite strains in this study were more sensitive to artemether-lumefantrine combination than any other drug combination used as reflected in Fig 1 and Table 2 and 3. The IC₅₀s ranged from (0.00000 - 12.47460) x 10⁴ nM.

The parasites were least sensitive to artemether-quinine combination with IC₅₀s ranging from (0.00000 – 5.92475) x 10⁴ nM. Five (5) parasite strains were absolutely resistant to artemether – lumefantrine and artemether – amodiaquine combinations. Seven (7) parasite strains were absolutely resistant to artemether – quinine. Artemether – amodiaquine had the lowest IC₅₀ range [(0.00000 – 4.73448) x 10⁴ nM] and artemether – lumefantrine the highest [(0.00000 – 12.47460) x 10⁴ nM]. The two parasite strains (from patients 6 and 7) that were sensitive to all single drugs were also sensitive to all the combined drugs.
Table 2: The antimalarial susceptibility of *P. falciparum* isolates from 17 patients who presented with uncomplicated malaria at Mulago Hospital to drug combinations. *** Refers to samples that showed absolute resistance.

<table>
<thead>
<tr>
<th>Patient</th>
<th>ART - LUM (nM) X10^4</th>
<th>ART - AQ (nM) X10^4</th>
<th>ART - QNN (nM) X10^4</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.00001</td>
<td>0.04823</td>
<td>0.07040</td>
</tr>
<tr>
<td>2</td>
<td>0.00001</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>3</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>4</td>
<td>0.69712</td>
<td>1.76646</td>
<td>***</td>
</tr>
<tr>
<td>5</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>6</td>
<td>0.00000</td>
<td>0.00000</td>
<td>0.00000</td>
</tr>
<tr>
<td>7</td>
<td>0.00000</td>
<td>0.00000</td>
<td>0.00000</td>
</tr>
<tr>
<td>8</td>
<td>0.20083</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>9</td>
<td>***</td>
<td>0.60643</td>
<td>0.40105</td>
</tr>
<tr>
<td>10</td>
<td>12.47460</td>
<td>0.49336</td>
<td>3.56751</td>
</tr>
<tr>
<td>11</td>
<td>0.64602</td>
<td>3.75367</td>
<td>0.47145</td>
</tr>
<tr>
<td>12</td>
<td>***</td>
<td>0.82251</td>
<td>3.20957</td>
</tr>
<tr>
<td>13</td>
<td>0.00688</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>14</td>
<td>***</td>
<td>0.08314</td>
<td>0.27132</td>
</tr>
<tr>
<td>15</td>
<td>0.44961</td>
<td>2.61060</td>
<td>***</td>
</tr>
<tr>
<td>16</td>
<td>1.36018</td>
<td>1.84635</td>
<td>1.96035</td>
</tr>
<tr>
<td>17</td>
<td>0.15019</td>
<td>4.73448</td>
<td>5.92475</td>
</tr>
<tr>
<td>Cutoff IC50 (nM)</td>
<td>0.70000</td>
<td>0.85000</td>
<td>0.50000</td>
</tr>
</tbody>
</table>
Table 3: Range of IC\(_{50}\) of the different drugs used to determine antimalarial susceptibility of *P. falciparum* isolates from 17 patients who presented with uncomplicated malaria at Mulago Hospital.

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC(_{50}) range (nM) (\times) 10(^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chloroquine</td>
<td>0.00000 – 25.69767</td>
</tr>
<tr>
<td>Artemether</td>
<td>0.00000 – 14.55405</td>
</tr>
<tr>
<td>Quinine</td>
<td>0.00000 – 4.29374</td>
</tr>
<tr>
<td>Amodiaquine</td>
<td>0.00000 – 4.97200</td>
</tr>
<tr>
<td>Lumefantrine</td>
<td>0.00000 – 26.82420</td>
</tr>
<tr>
<td>Artemether-Lumefantrine</td>
<td>0.00000 – 12.47460</td>
</tr>
<tr>
<td>Artemether-Amodiaquine</td>
<td>0.00000 – 4.73448</td>
</tr>
<tr>
<td>Artemether-Quinine</td>
<td>0.00000 – 59.24747</td>
</tr>
</tbody>
</table>

Below is the percentage sensitivity of the parasite isolates to the different drug and drug combinations used in the study.

Figure 1: The percentage sensitivity of *P. falciparum* isolates from 17 patients who presented with uncomplicated malaria at Mulago Hospital to antimalarial drugs and drug combinations.
CHAPTER FIVE
DISCUSSION AND CONCLUSIONS

5.1 Discussion

The current malaria treatment policy by the Ministry of Health in Uganda recommends the use of artemisinin based combination therapy (ACT) for uncomplicated malaria. The ACT in Uganda combines Artemisinin derivatives with either Lumefantrine or Amodiaquine or SP or Mefloquine.

In this study, the combination of artemether – lumefantrine was found to be more effective than that of artemether – amodiaquine and Artemether - quinine. This is in line with what Hasifa et al., 2006 found out during a clinical trial in Tororo- Uganda and some studies in Zanzibar (Martensson et al., 2005). However, the sensitivity to artemether – lumefantrine by the strains in this study is low compared to the curative rates noted in malaria trials in Uganda. The low sensitivity could be due to the pharmacokinetics of artemether in vivo in which it is converted to the more active derivative dihydroartemisinin, a state not achieved in vitro.

The strain sensitivity to artemether - quinine was the lowest in this study. This may suggest that there could be drug interactions leading to antagonism of the individual drug effects. The low sensitivity to artemether - quinine observed in this study does not however correlate with observations in other studies which indicate synergism in vitro (Gupta et al., 2002, Ekong and Warhurst,1990, Fivelman et al., 1999). Nevertheless, the effect of artemether – quinine in vivo could be different from all these observations since drug effects in vitro do not always correlate with in vivo effects.
In vitro results however, do not always depict in vivo outcomes. The way the body acts on drugs in vivo through aspects such as absorption, metabolism, elimination and immunity may modify the drug effects ultimately altering its activity. In immuno-suppressed individuals for example, treatment outcomes may be quite poor since the fight against infection is only effected by drugs, which may not be very competent without the involvement of the immune system.

Although the AMDP no longer recommends the use of monotherapies, drugs like chloroquine are still used by some individuals in Uganda. Chloroquine resistance has steadily risen over the past 20 years with recent studies indicating that chloroquine fails to clear parasites in up to between 50-80% of patients in East Africa (Brandling-Bennett et al., 1998; Sexton et al., 1988; Watkin et al., 1988, Bayoumi et al., 1989; Fowler et al., 1993, Premji et al., 1993, Wolday et al., 1995). This study indicates a further decrease in sensitivity to chloroquine since it is one of the least effective among the single drugs with sensitivity rates less than 50%.

In 1995, Nevill et al determined the sensitivity of P. falciparum isolates in Uganda to chloroquine and amodiaquine and found out that the isolates were significantly less sensitive to chloroquine than to amodiaquine. This is different from the results obtained in this study in which amodiaquine is slightly less active than chloroquine among single drugs probably due to cross-resistance. In vivo studies however report better treatment outcomes in amodiaquine combinations than chloroquine. This reflects some of the discrepancies between in vitro and in vivo studies. In Uganda, amodiaquine has not been widely used due to toxicity concerns and potential cross resistance with chloroquine (Oliaro et al., 1996). Amodiaquine is also associated with adverse events such as hepatotoxicity and blood dyscrasias when used for prophylaxis in travellers (Phillips-Howard and West, 1990). Recent
studies however suggest that amodiaquine is efficacious and safe for malaria treatment in areas of
high chloroquine resistance (Olliaro et al., 1996; Brasseur et al., 1999; Gorissen et al., 2000; Staedke
et al., 2001).

In this study, two (2) isolates were completely sensitive to all drugs that it was not possible to obtain
their IC\textsubscript{50} values. This implies that much as resistance has developed to most of the antimalarial
drugs, some individuals may actually respond to the drugs currently in use. Therefore, there’s need to
carry out a regional mapping of the sensitivity of malaria parasite so that antimalarial drugs can be
distributed appropriately in the country. Alternatively, a simple chloroquine resistance detection kit
could be developed to enable use of chloroquine in such cases putting into consideration the
availability and affordability of chloroquine.

5.2 Study limitations
The sample size was not big enough and therefore the results may not be used to project outcomes for
a large population. The strains in the study had not been typed. They were wild strains and so you
could not tell which kind you are working with in the study, whether for example chloroquine-
sensitive or chloroquine - resistant.

5.3 Conclusions
From the results of this study, the parasites were most sensitive to quinine among the single drugs
with lumefantrine and amodiaquine being the least active drugs. This indicates that quinine is still
effective as monotherapy for the treatment of uncomplicated malaria in Uganda.
Among the combined drugs, the parasites were most sensitive to artemether – lumefantrine and least sensitive to artemether – quinine. This implies that combining artemether and quinine may not be applicable for the treatment of uncomplicated malaria unless proved effective through further vigorous research.

5.4 Recommendations

Quinine can continue being used as monotherapy for the treatment of malaria in Uganda.

Artemether – lumefantrine combination is effective for the treatment of uncomplicated malaria and should therefore continue being used as a combined regimen. This is in accordance with the current antimalarial drug policy in Uganda.

Another study with a larger sample size should be carried out to obtain results that can be projected to the entire Ugandan population.

A regional mapping for the susceptibility pattern of *P. falciparum* be carried out to establish which areas (regions and/or races), respond to which drugs.

Another study should be conducted in order to identify individuals who respond to all drugs so that they can be followed up to establish why these individuals respond in this way. This could be as a result of the genetic make up of the individuals or of the parasite species they carry.
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  *Drug resistance Update*, **1**:3-9

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APPENDIX: INFORMED CONSENT FORM

Principle investigator:
Nakaziba Rebecca
Dept of Pharmacology and Therapeutics
Makerere University Medical School

Purpose of the study
The study is being carried out to determine the sensitivity of the malaria parasite
(Plasmodium falciparum) to the currently used antimalarial drugs.
If you are willing to take part in this study, you will be required to provide a blood
sample.

Study procedure
The study participants will provide a blood sample of about 4 mls, which will be taken
to the laboratory for further investigations.

Risks
You will feel slight pain while the blood is being drawn.

Benefits
You will receive treatment for malaria.
Joining the study is entirely voluntary and you may withdraw your participation at any
time without affecting your right to receive medical care.
There are no payments for participation in this study.
For further information contact
Nakaziba Rebecca
Department of Pharmacology and Therapeutics.
Tel 0712 528044
STATEMENT OF CONSENT

I, ......................................................, having been asked to participate in the study to determine the sensitivity of \textit{P. falciparum} to the current antimalarial drugs, declare that I clearly understand that

a) participation in this study is entirely voluntary and I can withdraw my consent at any time without affecting my right to medical care and

b) I am to provide a blood sample.

I therefore accept to participate in the study.

Signature/ thumbprint ...........................................
Date ..............................................................

I, Rebecca Nakaziba (Principal Investigator), declare that I have explained the purpose of the study to the above named patient and that to the best of my knowledge; he/she has understood and accepted to participate in the study.

Signature ...........................................
Date ............................................................