STUDIES ON MALARIA PREVALENCE, DRUG TREATMENT REGIMES AND INTENSITY OF CHLOROQUINE-RESISTANT *PLASMODIUM FALCIPARUM* IN ZARIA, NIGERIA

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DECLARATION

I declare that the work in this Thesis entitled "STUDIES ON MALARIA PREVALENCE, DRUG TREATMENT REGIMES AND INTENSITY OF CHLOROQUINE-RESISTANT *PLASMODIUM FALCIPARUM* IN ZARIA, NIGERIA" has been undertaken by me in the Department of Biological Sciences under the supervision of Dr. I.S. Ndams and Dr. P.A.Wuyep, Department of Biological Sciences. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at any university.

Name of Student	**Student**	**Date**

CERTIFICATION

This Thesis entitled ‘’STUDIES ON MALARIA PREVALENCE, DRUG TREATMENT REGIMES AND INCIDENCE OF CHLOROQUINE-RESISTANT *PLASMODIUM FALCIPARUM* IN ZARIA, NIGERIA” by BECHEMAGBOR ASHUTAKA meets the regulations governing the award of the Degree of Masters in Zoology of Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

(**Names and Signatures**)

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Chairman, Supervisory Committee **Date**

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Member, Supervisory Committee **Date**

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_____ _____

Dean, Postgraduate School **Date**

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ABSTRACT

This study was carried out to investigate the malaria prevalence and chloroquine-resistant *Plasmodium falciparum* among malaria patients in Zaria. Six hundred and seventy-eight (678) patients demographic and socio-economic information was obtained using a structured questionnaire. 5% Giemsa stained thick blood films from the patients were examined using light microscopy and the samples were also confirmed for positivity using Rapid Diagnostic Test cassettes. Sucrose-sorbitol density gradient centrifugation was carried out to obtain asexual stages of the parasite. The eluent were inoculated into a complete malaria culture medium to increase the parasite density/ml. Isolation of genomic DNA extraction of *Plasmodium falciparum* was carried out from the culture media using the standard phenol/chloroform extraction method. Mutations associated with CQ-resistance at codon 76 of *pfcrt* were analyzed using nested PCR/RFLP. The isolates were genotyped for pfcrt-76 since the presence of a mutation at this codon indicates that the parasite carries a resistant gene. Of the 678 samples examined, 159 were *Plasmodium* slide positive indicating an overall prevalence of 23.45%. Of the 425 females examined, 120 were malaria positive while 81 out of the 253 males were positive indicating prevalence rates of 28.35% and 32.01% respectively. Mean percentage prevalence among pregnant and non-pregnant women was 50% and 26.22% respectively. RDT showed 36 positive results out 159 microscopic samples (22.64%). DNA extraction from the 36 positive samples yielded no results. To this end, CQ still elicits its therapeutic effect despite previous reports on the parasite resistance to the drug. Standard Microscopy, RDT and PCR/RFLP should be employed in diagnosis, treatment and drug resistance surveillance respectively to curtail the advent dilemma.

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ABBREVIATIONS

CQ = Chloroquine

CQR – Chloroquine Resistant

CQS – Chloroquine Susceptible

CRPF – Chloroquine Resistant *Plasmodium falciparum*

DNA – Deoxyribonucleic Acid

DV – Digestive vacuole

EDTA- EthyleneDiamineTetra Acetic acid

K76T – position amino acid Codon for Threonine and Lysine

MCM – Malaria Culture Medium

MSP – Mefloquine Sulfadoxine Pyrimethamine

NAFDAC - National Agency for Food and Drug Administration Control

PCR – Polymarase Chain Reaction

pfcrt – *Plasmodium falciparum* Chloroquine Resistant Transporter

pfmdr – *Plasmodium falciparum* multi-drug resistant gene 1

RBM – Roll Back Malaria

RFLP- Restriction Fragment Length Polymorphism

RPMI 1640-Roswell Park Memorial Institute 1640

TBE- Tris-Boric EDTA

EDTA- EthyleneDiamineTetra Acetic Acid

DEFINITION OF TERMS

Drug resistance – is the ability of the parasite strain to survive and/or multiply despite administration and absorption of the drug in equal doses or higher than those recommended but within limits of tolerance of the subject.

Parasitaemia – the level of parasites found in blood.

Amplicon – products of Polymerase Chain Reaction (PCR).

Electrophoresis – in a technique for separation and analysis proteins and Nucleic Acid

Sensitivity – ability of a test to correctly give a positive result when an individual tested is truly diseased or infected.

Specificity – ability to test to give a negative result when an individual tested in truly not diseased or infected.

Predictive value – the probability that the result reflects the disease status.

Restriction Enzyme - Is a protein that binds to DNA molecule at a specific sequence and makes a double stranded DNA cut at or near a sequence

Primer - single stranded DNA between 20-50 nucleotides long, complementary to a short region on either side of the DNA template.

Taq-polymerase - a heat stable enzyme that drives or catalyses the synthesis of new DNA molecule

CHAPTER ONE

INTRODUCTION

1.0 BACKGROUND

Malaria remains one of the most devastating diseases of the developing world, killing 1-3million people and causing disease in 300-500 million people annually (WHO, 2003). *Plasmodium falciparum* is a blood-borne Apicomplexan parasite that affects persons living in sub-Saharan and other parts of the world (South East America, Asia). This parasite is responsible for most of the complication (Black water fever, gastrointestinal disturbance, hyperpyrexia, seizures, and hypoglycemia in pregnant women) associated with human malaria.

Antimalarial drug resistance is a major public health problem, which hinders the control of malaria. Drug resistance in malaria describes the knowledge about the problem and also outlines the current thinking regarding strategies to limit the advent, spread and intensification of drug-resistant malaria, thereby affecting challenges facing malaria control today (WHO, 2001). Resistance of *Plasmodium falciparum* to chloroquine, the cheapest and most used drug is spreading in almost all endemic countries. Chloroquine is a 4-aminoquinoline derivative of quinine, which was synthesized around 1934, has since been the most widely used antimalarial drug (Bruce-Chwatt, 1980; WHO, 2001). Chloroquine resistant *Plasmodium falciparum* has been described everywhere *Plasmodium falciparum* malaria is transmitted except for malarious area of central America, in the limited areas of the Middle East and Central Asia (Mockenhaupt, 1995). Again, many antimalarial drugs currently in use are closely related chemically and development of resistance to one can facilitate development of resistance to others (Basco, 1991).

The detection of resistance in *Plasmodium falciparum* to antimalarial drugs involves 4 basic methods that are employed routinely. This includes; in-vivo test, in-vitro test, animal model studies and molecular characterization (Bloland, 2001). Molecular techniques are currently the choice methods which have better promising advantages over the other three models. This molecular technique utilizes the Polymerase Chain Reaction (PCR) Restriction enzyme and Restriction Fragment Length Polymorphism (RFLP) to indicate the presence of mutation and the DNA Fragment encoding biological resistance to antimalarial drugs.(Plowe *et al.,* 1995). This technique has become a valuable surveillance tool for monitoring the occurrence, spread or intensification of drug resistance in chloroquine, sulfadoxine-pyrimethamine, cycloguanil, mefloquine, and atovaquone which are the only drugs with known molecular markers (WHO, 2001).

Drug resistance is the ability of a parasite strain to survive and / or multiply despite the administration and absorption of a drug in equal doses of higher than those recommended but within limits of tolerance of the subject (WHO, 1963). Antimalarial drug resistance in malaria has been classified,based on in-vivo:-

(i) RI-Initial clearance of the parasite but re-appearance within a month after onset of treatments.

(ii) RII -A reduction in parasitaemia after treatment but failure to clear the parasite with a subsequent rise in parasitaemia.

(iii) RIII -Severe form of resistance i.e. parasitaemia shows no significant change with treatment. (WHO, 1963; Cheesbrough,1985)

The gold standard drug for treatment of malaria was chloroquine, a 4-aminoquinoline derivative which has been characterized for its efficacy, low toxicity and

affordability (Fidock *et al.,* 2004). Inspite of the prevalence of chloroquine resistant *Plasmodium falciparum*, the drug is continuously used widely especially in sub-Saharan Africa. If new antimalarial drugs have to be deployed then it must meet the requirement of rapid efficacy, minimal toxicity and low cost since cost drives choice of drugs in most developing countries (Fidock *et al.,* 2004).

Chloroquine resistant falciparum malaria has been reported in Ibadan, South West Nigeria (Solako *et al.,* 1987 ; Sowunmi *et al.,* 1998; Ajayi *et al.,* 1998; Ademowo *et al*., 2002; Fehintola *et al.,* 2002; Happi *et al.,* 2003); Jos and Zaria, North Central Nigeria (Lege-Oguntoye *et al,,* 1989), Calabar, Eastern Nigeria (Ezedinachi *et al.,* 1992) and Sokoto, North Western Nigeria (Abdullahi *et al.,* 2003).

Chloroquine resistant *P. falciparum* was associated with specific point mutations in the *P. falciparum* chloroquine-resistant transporter (*pfcrt)* and *Plasmodium falciparum* multi-drug resistant (*Pfmdr- 1*) genes among children aged 1-12 years in Ibadan, Nigeria. Prevalence ranges of 53% and 40% with T76 allele of *pfcrt* and the Y86 allele of *Pfmdr-1* respectively, have been reported (Happi *et al.,* 2003). The acquisition of resistance by the malaria parasite is an evolutionary selection pressure and it is a stable trait in the parasite once it is acquired (Foote *et al.,* 1990; Wellems *et al.,* 1990).

A similar study on chloroquine resistant *P. falciparum* in Ilorin, Nigeria reveals that twenty-four patients (20%) showed RII response while 6 patients (5%) showed RIII response out of the 120 patients sampled with ages ranging from 6 months to 34 years. Hence, the finding suggests that there is a need for extra vigilance of Chloroquine-Resistant *P. falciparum* (CRPF) malaria in general and malnourished children in particular

if potentially serious complications are to be averted (Olanrewaju *et al.,* 2001). In quantifying the economic burden of malaria in Nigeria using the willingness to pay approach, it shows that the disease imposes great burden on the society and it has an adverse effect on the physical, mental and social well being of the people and on the economic development of the nation due to poverty (Ayodele *et al.,* 2007).

1.1 STATEMENT OF PROBLEM

Chloroquine is one of the drugs of choice for the treatment of Malaria in Nigeria and still remains the most widely used antimalarial drug in the West African sub-region (Bruce-Chwatt, 1980; Elebiyo, 2002; F.M.H, 2009) but Chloroquine resistance has been reported in many countries in sub-saharan Africa. Thus, there is the need to update and address the situation using the newly developed and validated molecular tools used for surveillance to provide valuable information.

1.2 HYPOTHESES

(i) There is no chloroquine-resistance in *Plasmodium falciparum* in the study area**.**

(ii) There is no association between chloroquine-resistant *Plasmodium falciparum* with age**.**

(iii) There is no significant association between chloroquine-resistant *Plasmodium falciparum* with sex.

(iv) There is no significant association between chloroquine-resistant *Plasmodium falciparum* with level of parasitaemia

(v) There is no significant difference between Microscopy and Rapid Diagnostic Test

1.3 AIM

To study the prevalence of chloroquine-resistant *Plasmodium falciparum* in falciparum malaria patients in Zaria.

1.4 OBJECTIVES

The objectives of the study are to:-

i. Determine chloroquine-resistant *Plasmodium falciparum* mutation in falciparum malaria patients in Zaria.

ii. Determine association between chloroquine-resistant *Plasmodium falciparum* with age.

iii. Determine association between chloroquine-resistant *Plasmodium falciparum* with sex.

iv. Determine association between chloroquine-resistant *falciparum* with parasitaemia.

v. Determine comparison between Microscopy and Rapid Diagnostic Test.

1.5 SIGNIFICANCE

- This study will provide baseline data that will serve as a heuristic tool for stakeholders involved in malaria eradication to meet their objectives of the Roll Back Malaria programme.
- Provide also a guide or a rationale for the sales of antimalarial drugs and treatment of malaria especially *Plasmodium falciparum* from epidemiological surveillance data for the update on drug policies.

- Contribute relevant data to the Local Government, Federal Ministry of Health and their developmental agencies on the drug status in the region.
- It would also go a long way to remind practitioners on the current status of the drug in the region in annual workshops or seminars.

1.6 JUSTIFICATION

(i) Sensitivity and specificity are of crucial importance in any identification method, because false negative and false positive results would expose the subject or individual to unnecessary drug administration.

(ii) Microscopy remains the cheapest and commonly used method for diagnosis of malaria. This relies on the microscopic examination of stained blood films. Its limitation when performed even by an expert is time consuming and sensitivity of detection limited when parasitaemia is low. (Coleman *et al.,* 2002). Hence, improved techniques such as molecular technique, immunochromatographic test (Rapid Diagnostic Test) should be employed for detection.

(iii) Resistance to *P. falciparum* had been reported in Zaria (Lege-Oguntoye *et al*., 1989; Dalti, 2002).

(iv) PCR/RFLP is currently in use and validated methods to establish resistance in malaria.

(v) Data from Microscopy/In-vitro, In-vivo and Animal models are not reliable.

CHAPTER TWO

LITERATURE REVIEW

2.0 THE PARASITE

Morphological differences among the human malaria parasite make diagnosis possible. Infected red blood cells have a ring of bluish cytoplasm with a dot-like nucleus of red chromatin. The ring forms of early trophozoites of the *Plasmodium falciparum* as well as the gametocytes are seen in peripheral blood. The immature gametocytes are elliptical in shape or crescent-shaped. The trophozoites (ring forms) are 1/5 of red blood cell with a very delicate ring, fine dots that are frequently two and non-pigmented at this stage (Symth, 1996). The fine structure of the parasite reveals cellular component, which acts as target site for most antimalarial drug. Some of the cellular components are the digestive vacuole, lysosomes. The organism is oval in shape, and surrounded by a pellicular complex. The pellicle consist of an outer and two closely aligned inner membranes. Beneath the inner membrane is a row of subpellicular microtubules originating from the distal polar ring of the apical complex.

There is a circular cytostome situated laterally which is involved in ingestion of host cytoplasm. The nucleus lacks a nucleolus. The cone shaped apical end contains three polar rings, two rhoptries and several micronemes. These structures aid penetration into the erythrocytes. The merozoites are transformed to trophozoites. The exact process of digestion of cytoplasm through the cytostome varies among other species but food vacuoles in which digestion occurs are formed. The digestion process is characterized by formation of particles of the malarial pigment haemozoin. (Aikawa and Seed, 1980; Symth, 1996; Wikipedia, 2007)

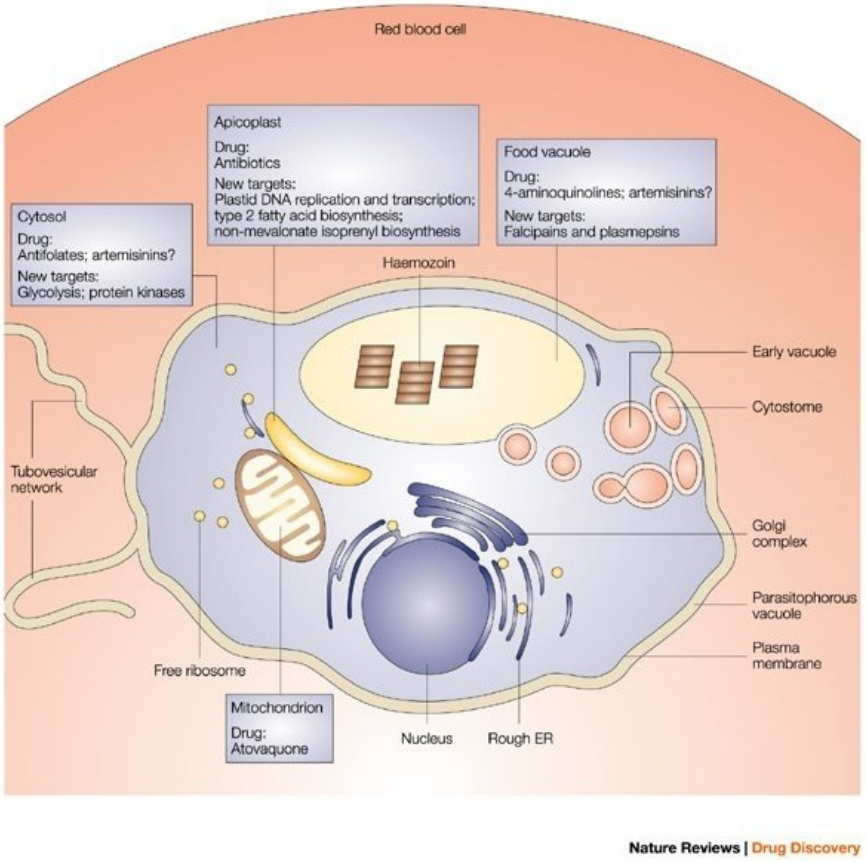


Fig 2.1a Ultra structure of *Plasmodium falciparum*

Representation of an intra-erythrocytic Plasmodium falciparum trophozoite, highlighting key parasite intracellular compartments and the site of action of some of the major classes of antimalarial drugs. (www.nature.com/ *Plasmodium falciparum*)

2.1 THE BIOLOGICAL MEMBRANE OF *PLASMODIUM FALCIPARUM* AND DIGESTIVE VACUOLE.

A membrane transport protein (or simply transporter) is a protein involved in the movement of ions small molecules, or macromolecules, such as another protein across a biological membrane. Transport proteins are integral membrane proteins; that is they exist within and span the membrane across which they transport substances. The proteins may assist in the movement of substances by facilitated diffusion or active transport. The mechanism of action of these proteins is known as carrier-mediated transport.

There are two forms of carrier-mediated transport, active transport and facilitated diffusion. (Wikipedia, 2009)

2.2 CHLOROQUINE

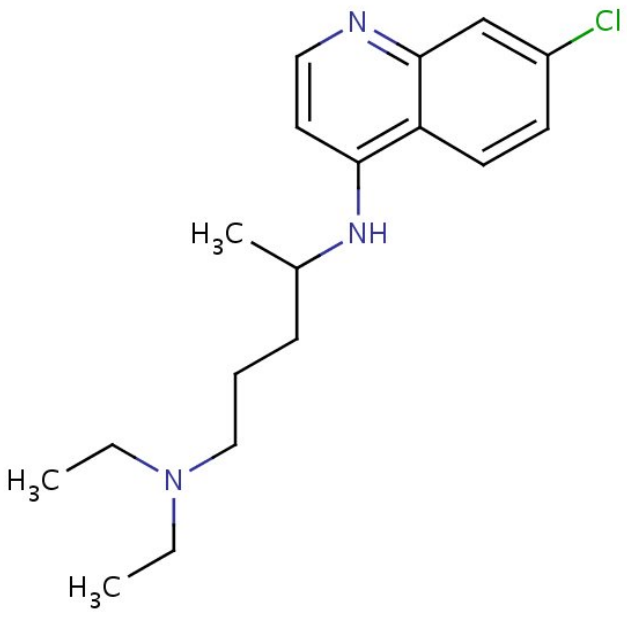


Fig 2.2.0a: 7-Chloro-4-(4-diethylamino-1-methylbutylamino)quinoline

Common Names: Chingamin, Chingamin phosphate, Chlorochin, Chloroquin diphosphate Chloroquina, Chloroquine diphosphate, Chloroquine hydrochloride, Chloroquine phosphate, Chloroquine sulfate, Chloroquine sulphate, Chloroquinum, Cholarquine, Choroquin sulfate, Quinagamin

Brand Names: Aralen, Avloclor, Arquin, Chinophen, Delagil, Fetoquin,Vinoquine. Malarex, Kalquin, Baraquine. (www.drugs.com/chloroquine)

The formulations are usually as tablet or syrups. (Wernsdorfer, 1982; Wikipedia, 2007). The quinolines include some of the most common antimalarial drugs: type 1 quinolines (the 4-aminoquinolines chloroquine and Mannich base compounds amodiaquine and pyronaridine) and type 2 quinolines (the aryl-amino alcohols quinine and quinidine, mefloquine, halofantrine, and lumefantrine). The 2 types interact differently with their putative target and show an inverse association with respect to parasite susceptibilities. Type 1 drugs essentially inhibit the detoxification of heme (the byproduct of digestion of hemoglobin) as is the case with chloroquine.

Therapeutic Dosage

Adults:

Malaria treatment

Oral: 600 to 900 mg the First day, then 300 mg daily

I.M.: 200 to 300 mg/day for three to five days.

Malaria prophylaxis

Oral: 100 mg/day or one dose of 300 mg weekly for six Weeks.

Children:

Malaria treatment

Oral: 10 mg/kg as loading dose, then 5 mg/kg/day for four days.

IM: is not recommended in children.

Malaria prophylaxis

Oral: age below 1 year: 25 mg every two days; age over 1 year; 25 to 50 mg per-day or; 5 mg/kg one dose weekly. (www.drugs.com/chloroquine.html; FMH, 2009)

2.2.1 Physiology of the Mechanism of Chloroquine-resistance to *Plasmodium falciparum*

Chloroquine is a 4-aminoquinoline drug, long used in the treatment or prevention of malaria. Chloroquine is a lysosomotropic agent i.e. it accumulates preferentially in the lysosomes of cell in the body. The pka for the quinoline nitrogen of chloroquine is 8.5 implying 10% is deprotonated at physiological pH as calculated by the Henderson-Hasselbach equation. Since the deprotonated form is more membrane permeable than the protonated form, it results to quantitative trapping of the compound in lysosomes.

To add the lysosomotropic character is known to account for the antimalarial activity of drug in the acidic food vacuole of the parasite thereby affecting essential process within the parasite. (Elebiyo, 2002; Wikipedia, 2007)

2.2.2 Quinolines.

Resistance to chloroquines is multigenic and epistatic and affects drug accumulation in the parasite food vacuole and not the showing drug target in the parasite. There are evidence from isolates around the world that chloroquine resistance correlates with a single amino acid mutation (Lys→Tyr) at codon 76 (K76T) of the parasite *pfcrt* gene (Fidock *et al*., 2000). Satellite *pfcrt* mutations occur and the patterns tend to vary in different geographic areas (Wellems and Plowe, 2001). To add, polymorphism in *pfmdr1,* which encodes the *P. falciparum* P glycoprotein homologue 1, may modulate resistance in parasites harboring the *pfcrt* mutation.

The efficacy of chloroquine against the parasite declined as resistant strains of the parasite evolve thereby neutralizing the effect of the drug. Chloroquine resistant cells efflux chloroquine 40 times the rate of chloroquine sensitive cells. This is related to a number of mutations that trace back to transmembrane proteins of the digestive vacuole including an essential mutation in the *pfcrt* gene. This mutated protein actively pumps chloroquine from the cell. Resistance has also been conferred by reducing the lower transport activity of the intake mechanism. (Plowe, 2005).

2.2.3 Mechanism of action of chloroquine

The parasite produces the toxic and soluble molecules haem. This consists of a porphyrin called Fe (II)-protoporphyrin 1 x (FP). To avoid destruction of the molecule, the parasite polymerizes haem to form haemozoin (non-toxic) Haemozoin collects in the digestive vacuole as insoluble crystals. Chloroquine enters the red cell inhabiting the plasmodium falciparum and digestive vacuole by simple diffusion. As a result, chloroquine

becomes protonated (CQ^{2+}) as the digestive vacuole is known to be acidic. The drug caps haemozion molecules to prevent polymerization to haem. The binding of the drug with the haemozion forms a complex known as FP- chloroquine complex. This complex becomes toxic to the cell and disrupts membrane function. Thus, the parasite drowns in its own metabolic products. (Uhlemann and Krishna, 2005; Wikipedia, 2007). The mechanism of action of antimalarial involves prevention of haemoglobin digestion. Here, the 4-aminoquinoline interferes with the essential process of haemoglobin digestion.

The drug (chloroquine) binds to the haem complex ferroproptoporphyrin- x haem, which is a by-product of haemoglobin degradation that is capable of killing the parasite. Chloroquine resistance is characterized by reduced cellular drug accumulation that is expressed phenotypically. Hence structural changes in target enzymes are responsible for easily developed resistance to antifolates and anti mitochondrial. Drug efflux through the multi drug resistance membrane protein and the production of a protein competing with drug for the target hemin is believed to be responsible for resistance to blood schizontocides. (Symth, 1996; Plowe, 2005; Warhurst *et al.,* 2005; Uhlemann and Krishna, 2005; Wikipedia, 2007).

The actual mechanisms accounting for resistance are of 3 major categories.

i. Inactivation of drug by the parasite

ii. Decreased penetration of drug into the cell

iii Alteration of parasite metabolism so as to eliminate the step(s) that was previously susceptible to the drug.

Hemoglobin metabolism

In the erythrocytic stage of the parasite's life cycle, it uses intracellular hemoglobin as a food source. The protein is broken down into peptides, and the heme group is released and detoxified by biocrystallization in the form of hemozoin. (Hempelmann, 2007)

Carbohydrate metabolism

During erythrocytic stages, the parasite produces its energy mainly through anaerobic glycolysis, with pyruvate being converted into lactate.

Genes encoding for the TCA cycle enzymes are present in the genome, but it is unclear whether the TCA cycle is used for oxidation of glycolytic products to be used for energy production, or for metabolite intermediate biosynthesis. It has been hypothesized that the main function of the TCA cycle in *P. falciparum* is for production of succinyl-CoA, to be used in heme biosynthesis. Genes for nearly all of the pentose phosphate pathway enzymes have been identified from the genome sequence.

Protein metabolism

It has been hypothesized by Gardner *et al.* (2002) that, the parasite obtains all, or nearly all, of its amino acids by salvaging from the host or through the degradation of hemoglobin. This is supported by the fact that genomic analysis has found no enzymes necessary for amino acid biosynthesis, except for glycine-serine, cysteine-alanine, aspartate-asparagine, proline-ornithine and glutamine-glutamate interconversions. Much of this digestion occurs

within the digestive vacuole. Multiple enzymes are involved in this process including four distinct plasmepsins (aspartic acid proteases).

Nucleotide metabolism

Plasmodium falciparum is unable to biosynthesize purines. Rather, the parasite is able to transport and interconvert host purines. On the other hand, the parasite can produce pyrimidines *de novo* using glutamine, bicarbonate, and aspartate. (Garndner *et al*., 2002)

2.3.1 Drug Resistance

Drug resistance in malaria is the ability of the parasite strain to survive and /or multiply despite the administration and absorption of a drug in equal doses or higher than those recommended but within limit of tolerance of the subject. (WHO, 1963). Changes in the genetic susceptibility of the vector-borne disease and the vector have a serious effect on the ability of public health system to adapt to the changing effect. (Bjorkman and Philips-Howard, 1990; Curtis and Kasim 1993; Coetzee *et al*., 1999).

Resistance gene continues to be selected in malarial thereby causing resurgences in the disease incidence. (Malakooti *et al.,* 1998). The international spread of multiple drug-resistance malaria in several developing countries has been due to population movement. Thus drug resistance needs to be managed in order to control the dilemma. (Omumbo *et al.* 2002). The emergence of resistance gene e.g. mdr-like gene product of *Plasmodium falciparum* in resistance to quinoline-containing compound e.g. Chloroquine, mefloquine, primaquine etc is not clear. Thus, the resistance of *Plasmodium falciparum* to many other therapeutic agents is an increasing problem in most endemic areas. (Basco *et al*.,1995).

Ezedinachi *et al.,* (1999) reported that a low-dose MSP (Mefloquine, Sulfadoxine-

Pyrimethamine contribution was significant more efficacious with faster fever and parasite clearance times than chloroquine in Eastern part of Nigeria where there is chloroquine resistance *Plasmodium falciparum* malaria. The study showed a low –dose MSP response rate of 95% and 91% for presumptive and in vitro respectively while chloroquine showed 82% and 66% in the two groups respectively.

2.3.2 Levels of Resistance in Antimalarial Drugs

Resistance mutation occurs both in schizogony and sporogony due to shifting in character progressively towards high-grade resistance i.e. effects of point mutation. Classification of chloroquine resistance by WHO, 1963 from in-vivo test is as follows:-

i. RI, following treatment, parasitaemia clears but recrudescence occurs.

ii. RII, following treatment, there is reduction in parasitaemia but no clearance.

iii. RIII, following treatment, there is no reduction of parasitaemia.

2.3.3 Pathogenesis of Drug Resistance Malaria

Antimalarial activity of CQ is thought to occur in the digestive vacuole of the intra-erythrocytic stage of *Plasmodium falciparum*, where the drug interferes with the polymerization and detoxification of haem molecules released by digestion of haemoglobin. Proposals for CQR Mechanism include: Altered CQ uptake or efflux at the cytoplasmic membrane of the intra-erythrocytic parasite; Altered H^+ flux or CQ uptake at the parasite DV membrane; Reduced CQ access to its receptor, hematin (formed in the DV upon haemoglobin digestion); Increased glutathione mediated detoxification of CQ – haematin complexes.

Again, the molecules that have been said to be involved in the proposal mentioned above ,it includes P-glycoproteins, a Cl-channel regulator, a Na^+/H^+ exchanger, a DV/H^+ pump are factors that reduce CQ access to hematin and glutathione S-transferase involve in haemoglobin detoxification (Martin *et al.,*1987; Martiney *et al.,* 1995; Bray *et al*., 1998; Ginsburg *et al.,*1998).

The transmembrane protein *pfcrt* is located on the parasite digestive vacuole, the site of CQ action where there is acidification with the *pfcrt* point mutations. These mutation in *pfcrt* eventually results to CQ flux or reduced drug binding to hematin through the DV/pH effect. To add the identification of *pfcrt* facilitates molecular investigation into the mechanism of *P. falciparum* CQR as well as in epidemiological surveys of CQR strains in malarious regions.

However, the rate of change of frequency of drug resistant in the parasite population is a function of the proportion of host treated with drugs and the parasite transmission rate. (Mackinnon and Hastings, 1998). (Fidock *et al.,* 2000) reported that K76T and A220S (the amino acid in *pfcrt*, by position) that confer chloroquine resistance plays an important role in chloroquine resistance in subjects.

2.3.4 Transmission of resistance.

The spread of resistance from one parasite to another requires that the sexual forms (gametocytes) occur and infected anopheline mosquitoes, and that the resistance genotype is conserved during the sexual cycle. Drug treatment affects formation and infectivity of gametocytes. Levels of gametocytes are high after treatment with Sulfadoxine-pyrimethamine, whereas artemisinin derivatives reduce carriage of gametocytes, even

when combined with pyrimethamine-sulfadoxine (Adjuik *et al.,* 1997). The infectivity of gametocytes from patients who experienced failure of pyrimethamine-sulfadoxine and chloroquine treatment is increased, and gametocytes from cases of treatment failure may carry resistant genotypes (Butcher, 1997; Sutherland *et al*., 2002;). Genetic rearrangements take place in the midgut of the mosquito during the sexual life cycle of the parasite, potentially involving gametocytes of different origins. When the rates of mutated parasites are low, infections are polyclonal, and the prevalence of malarial infection remains high (i.e. mosquitoes can acquire infections from several subjects), resistance genes tend to be lost through recombination of different genotypes, and resistance is thinned movements of human populations also play a role. (Hill *et al.*, 1995)

For pyrimethamine-sulfadoxine, there is molecular evidence of a single origin of resistant alleles in Southeast Asia, which were originally imported into East Africa and are now spreading across the African continent (Roper *et al*, 2004). Conversely, chloroquine resistance linked to *pfcrt* (encoding a putative transporter located in the membrane of the parasite digestive vacuole) evolve independently in different areas, through multiple evolutionary pathways (Mehlotra *et al.*, 2001).

2.3.5 How mutations in *pfcrt* confer resistance

Mutations in *pfcrt* are believed to confer chloroquine resistance by reducing the amount of chloroquine accumulated by the parasite. The bulk of the intraparasitic chloroquine is believed to be concentrated within the Digestive vacuole (DV). This, combined with the DV membrane location of *pfcrt* is consistent with the view that mutations in *pfcrt* cause chloroquine resistance by reducing the drug concentration in the

DV. A single study actually provided evidence for reduced accumulation of CQ in the DV of CQR parasites. (Saliba *et al*., 1998). How *pfcrt* might exert such an effect on DV/CQ concentration is a subject that is open or unclear. The first is that the reduced partitioning of CQ into the DV of CQR parasites is a consequence of alterations in the pH of the DV (pH_{DV}). Secondly, it is also a consequence of the transport of CQ out of the DV.

2.3.6 Outcome of Treatment in the Presence of Drug Resistance

A key aspect in determining the efficacy or failure of a drug is the time during which drug levels exceed maximum parasiticidal concentrations. The shorter the time, the sooner positive rates of parasite multiplication occur and the sooner parasites and clinical symptoms reappear (Price and Nosten, 2001). Initially, the median time to recrudescence will get progressively shorter.

Appropriate duration of follow-up, proportional to the residence time of the drug under study, is therefore crucial to capture these late-recrudescent infections. As the levels of resistance worsens, treatment failures manifest progressively. The drug produces slower clinical and parasitological responses and increased gametocyte carriage, and patients need more treatment courses per episode. However, parasites and symptoms never clear (R2 response), and eventually there is no response to treatment at all (R3 response). These set backs prone the patients at risk of severe manifestations, such as anemia, further complications, and death (Trape *et al.,* 2002). At the population level, there will be more clinical episodes over time and an increasing prevalence thus more treatments required, severe complications, anemia, and mortality, and greater treatment and indirect costs to patients, communities, and systems (Anonymous, 2004). The ability to quantify these

effects in a timely fashion is important to establish the correct treatment policies and thus controlling malaria.

2.3.7 Implications of Drug-Resistant Malaria

It is however important to distinguish between drug resistance and treatment failure, as well as between real failure and reinfection. Treatment may fail for different reasons, such as poor compliance, wrong dosage, poor absorption (e.g., because of vomiting), lack of immunity, and resistance of the parasite. Resistance can be defined as the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in recommended (or higher) doses. Resistance can be otherwise described as a shift in the concentration-effect (dose-response) relationship toward higher concentrations to inhibit parasite growth. (White, 1997)

2.4 FACTORS THAT AFFECT DRUG RESISTANCE IN FALCIPARUM MALARIA

Several factors other than pharmacologic properties of the drug influence the emergence of drug resistance strain.

i. The way a drug is used- i.e. therapy is directed against larger populations of parasites with greater chance for resistant mutants than use in suppression against low number of parasites.

ii. Frequency of strain exposure to a drug in either the same or successive subjects. Such that, frequent exposure would favor selection when recognizable resistance depends on a series of mutations.

iii. The status of acquired immunity in the host: That is, the threshold of an effective drug is commonly lower in semi-immune individuals than in non-immune subjects.

iv. The species of parasite- This is particular in resistance to 4-aminoquinolines by *Plasmodium falciparum*. Again resistance to chloroquine in the parasite may carry a repressed gene or genes for the production of enzymes that will operate the escape route until such times as mutants arise that can protect the parasites by some other mechanism. (Peters, 1969; Bloland, 2001; WHO, 2003).

Several factors contributing to the advent, spread, and intensification of drug resistance exist, although their relative contribution to resistance is unknown. Factors that have been associated with antimalarial drug resistance include different issues as human behaviour, vector and parasite biology, pharmacokinetics, and economics. Conditions leading to malaria treatment failure may also contribute to the development of resistance. Hence, there is a linear relationship between treatment failure and drug resistant.

2.4.1 Biological influences on resistance

Data on the response of sensitive parasites to antimalarial drugs *in vitro* and the pharmacokinetic profiles of common antimalarial drugs, there is thought to always be a residuum of parasites that are able to survive treatment (Wernsdorfer,1991). Under normal circumstances, these parasites are removed by the immune system (non-specifically in the case of non-immune individuals). Factors that decrease the effectiveness of the immune system in clearing parasite residuum after treatment also appear to increase survivorship of parasites and facilitate development and intensification of resistance. This mechanism has been suggested as a significant contributor to resistance in South-East Asia, where

parasites are repeatedly cycled through populations of non-immune individuals (Verdrager,1986).

The nonspecific immune response of non-immune individuals is less effective at clearing parasite residuum than the specific immune response of semi-immune individuals. The same mechanism also explains poorer treatment response among young children and pregnant women (White, 1997).

The prevalence of malnutrition among African children under 5 years has been estimated to be 30% and an estimated 4 - 5 million children are expected to be infected with HIV (UNICEF, 1998). It has been proven that malnutrition or HIV infection plays a significant role in facilitating the development or intensification of antimalarial drug resistance. The prevalence of these illnesses could pose a tremendous threat to existing and future antimalarial drugs. Some characteristics of recrudescent or drug resistant infections appear to provide a survival advantage or to facilitate the spread of resistance conferring genes in a population (Mehlotra *et al.,* 2001).

Handunetti *et al.,* 1996 in their study posited that, patients experiencing chloroquine treatment failure had recrudescent infections that tended to be less severe or even asymptomatic. There is evidence that certain combinations of drug-resistant parasites and vector species enhance transmission of drug resistance, while other combinations inhibit transmission of resistant parasites. In South-East Asia, two important vectors, *Anopheles stephensi* and *Anopheles dirus*, appear to be more susceptible to drug-resistant malaria than to drug sensitive malaria (Wilkinson et al., 1976; Sucharit, 1977). In Sri Lanka, a study found that patients with chloroquine-resistant malaria infections were more

likely to have gametocytaemia than those with sensitive infections and that the gametocytes from resistant infections were more infective to mosquitoes (Handunnetti *et al.,* 1996).

Conversely, in Haiti, some malaria vectors may be somewhat resisting control refractory to drug-resistant malaria, which may partially explain the numerous chloroquine sensitivity that remain in the world in spite of very similar human populations and drug pressure. Many antimalarial drugs in current usage are closely related chemically and development of resistance to one can facilitate development of resistance to others. Chloroquine and amodiaquine are both 4-aminoquinolines and cross-resistance between these two drugs is well known (Basco*,* 1991*;* Hall *et al.,* 1975). Development of resistance to mefloquine may also lead to resistance to halofantrine and quinine. Antifolate combination drugs have similar action and widespread use of sulfadoxine-pyrimethamine for the treatment of malaria may lead to increased parasitological resistance to other antifolate combination drugs (Sutherland *et al.,* 2002). There is an interesting theory that development of resistance to a number of antimalarial drugs among some falciparum parasites produces a level of genetic plasticity that allows the parasite to rapidly adapt to a new drug, even when the new drug is not chemically related to drugs previously experienced.

The underlying mechanism of this plasticity is currently unknown, but this capacity may help explain the rapidity with which South-East Asian strains of falciparum develop resistance to new antimalarial drugs. Unfortunately, the same factor may increase the likelihood of resistance developing due to prolonged elimination periods. The relative

contribution of low compliance versus use of long half-life drugs to development of resistance is not known. (Rathod *et al.,* 1997).

Parasites from new infections or recrudescent parasites from infections that did not fully clear was exposed to drug blood levels that are high enough to exert selective pressure but are insufficient to provide prophylactic or suppressive protection (Wernsdorfor, 1991). When blood levels drop below the minimum inhibitory concentration (the level of drug that fully inhibits parasite growth), but remain above the EC5 (the concentration of drug that produces 5% inhibition of parasite growth), selection of resistant parasites occurs.

However, the interpretation of this and its implications for development of resistance has variously been described as resistance being more likely in low-transmission environments (Paul, 1995; Paul, 1998), high-transmission environments (Babiker and Walliker,1993; Mackinnon, 1997; Mackinnon and Hasting, 1998), or either low- or high- but not intermediate-transmission environments (Hastings, 1997, Mackinnon, 1998). This relationship between transmission intensity and parasite genetic structure is obviously complex and subject to other confounding/contributing factors (Hastings and Mackinnon, 1998).

Certainly, the rate at which resistance develops in a given area is sensitive to a number of factors beyond mere intensity of transmission (such as initial prevalence of mutations, intensity of drug pressure, population movement between areas, the nature of acquired immunity to the parasite or its strains), but that reducing the intensity of

transmission will likely facilitate prolonging the useful life span of drugs (Mackinnon and Hastings, 1998; Molyneux, 1999).

2. 4.2 Programmatic influences on resistance

Programmatic influences on development of antimalarial drug resistance include overall drug pressure, inadequate drug intake (poor compliance or inappropriate dosing regimens), pharmacokinetic and pharmacodynamic properties of the drug or drug combination, and drug interactions (Wernsdorfor, 1994). Additionally, reliance on presumptive treatment can facilitate the development of antimalarial drug resistance.

Development resistance due to drug pressure especially that exerted by programmes utilizing Mass Drug Administration (MDA), probably has the greatest impact on development of resistance (Payne, 1988; Wernsdorfor, 1994). Studies by Ettling *et al*., 1995 suggested that resistance rates are higher in urban and peri-urban areas than rural communities, where access to and use of drug is greater. Misinterpretation, over proper dosing regimen has been described. Windstanley, 2001 findings revealed that the use of presumptive treatment for malaria has the potential for facilitating resistance by greatly increasing the number of people who are treated unnecessarily but will still be exerting selective pressure on the circulating parasite population. In some areas and at some periods of the year, the number of patients being treated unnecessarily for malaria can be very large. Concurrent treatment with other drugs can increase the likelihood of treatment failure and may contribute to development of drug resistance. Similarly, concurrent illness may have an influence, with regards to malnourishment.

Shakoor *et al.* (1997) reports reveal that drug quality has also been implicated in ineffective treatment and possibly drug resistance. Either through poor manufacturing practices, intentional counterfeiting, or deterioration due to inadequate handling and storage. Again, drugs may not contain sufficient quantities of the active ingredients. In an analysis of chloroquine and antibiotics available in Nigeria and Thailand, between 37% and 40% of samples assayed had sub-standard content of active ingredients, mostly from poor manufacturing practices. Another study in Africa found chloroquine stored under realistic tropical conditions lost at least 10% of its activity in a little over a year.
(Ballereau, 1997)

2.4.3 Social and Economic Consequences of Drug Resistant Malaria

Different movement pattern of people around the world affects the distribution and incidences of malaria too where people can either be carriers of the pathogen into new environment or is transmitted to an uninfected individual through blood transfusion whereby the new susceptible are usually naive to the disease leading to a high mortality and morbidity rates (Gillett, 1989).

From the three malaria epidemiological zones in Nigeria i.e. the forest, Grassland and Savannah two levels of resistance have been reported thereby indicating the need to monitor the situation. The impacts of Global change to vector –borne disease as well its implication in drugs resistance also affects almost every aspect of human population (Colwell *et al.,* 1998). Philip (2001) reported that changes in human population growth, modification of the environment for agricultural purpose have important influences on malaria, for example, the development of resistance both in the vector and pathogen and

the declining of public health systems, sanitation are a major contribution factors to the global problem of malaria.

2.4.4 Impact of Drug Resistance to the Control of Malaria

Drug resistance in parasites aggravates the burden of malaria. The dimension of the problem is due mainly to the limited armamentarium of drugs used thus far to treat malaria and to policies and practices constrained by limited resources. All drugs in use are affected except, artemisinin derivatives. Recently, artemisinin resistance has been reported by Noedl *et al*., 2009. The scale and impact of resistance has been underestimated, leading to the continued use of failing drugs, which contributes to the rise in resistance and increased morbidity and mortality due to malaria. Pharmacological, epidemiological, and operational aspects favor the development and spread of resistance. Though the problem poses several challenges, much can be done to reverse the course of events: by adopting adequate tests to assess resistance, encourage and sustain development of new drugs, protect drugs against resistance through use of combinations, expand access to prompt and effective treatment, and promote evidence-based policies and sensible practices (Olliaro, 2005).

CHAPTER THREE

METHODOLOGY

3.1 STUDY AREA

The study area was carried out in Zaria and blood samples were collected from the consenting patients presented with malaria to the University Sick Bay, Main Campus, ABU Kongo Campus and Jama'a Hospital. All molecular work on the samples was at the ABU Centre for Biotechnology Research and Training. Zaria is situated at Latitude 11° 25″ North and Longitude 4° 27″ East of Nigeria (The World Gazetteer, 2007).

3.2 MATERIALS AND METHOD

Reagents: Ethidium Bromide, Agarose, Taq polymerase, M_gCI_2, dNTPs, l0X PCR Buffer, Heparinized tubes, 95% Alcohol, Cotton wool, Primers (*pfcrt* P_1, *pfcrt* P_2, *pfcrt* D_1, *pfcrt* D_2), lancets, deinozised distilled water, Running buffer, Loading dye (0.025%bromophenol blue,7%sucrose,10%Glycerol,Tris-Hcl and distilled water), *Apo*1 (*Athrobacter protophormiae*), 1kilobase DNA Marker, RPMI 1640 (Liquid without L-glutamine and HEPES buffer, pH 7.4), Glucose 20% (20g/100ml), 10mM Hypoxanthine,100mM L-glutamine, HEPES 1M (25mM), 7.5% $NaHCO_3$, 50mg/µl Gentamycin sulfate, Heparin 5000 i.u/µl, or Citrate phosphate, Infected RBC (10% parasitaemia) , Phosphate Buffered Saline (pH 7.2), 5% Saponin, 500cc Human blood Rh^+ A solution, Lysis buffer, 40mM Tris-Cl (pH 8.0), 80mM EDTA (PH 8.0), Proteinase K, Phenol equilibrated with 0.1M Tris-Cl (pH 7.0), Chloroform, Rnase, 3M Sodium acetate (pH 5.0), 70% Ethanol, TE buffer (10mM Tris-Cl, 1mM EDTA), 2% Giemsa powder, in phosphate buffer, Sorbitol, Sucrose, Washing medium (Tris-Hank).

Equipment: Sterile Blood Lancets, 5ml Hypodermic syringes, eppendorf Biophotometer, 0.5-1000µl Micropipettes, 250µl Eppendorf test tube, PCR Machines (Techne, model FTGEN5D), Horizontal Electrophoretic tank, PCR Laminar flow preparation station, Top weighing Balance(Model IR-30),Waterbath,100µl-1000µl micropipette tips, Microwave oven (Hinari, Model Mx 310 TSCL) ,UV-Transilluminator(TFX –35M), Digital Camera, Eppendorf Centrifuge(Model 5417R), Water bath, pH meter

3.2.1 Ethical Consideration

This study obtained approval from the Department of Biological Sciences, A.B.U, Zaria and the Director of the hospitals of the three study sites.

3.2.2 Administration of Questionnaire

A structured questionnaire was used to obtain each patients biodata such as age, sex, educational status occupation, treatment profile with antimalarial drugs, history of pregnancy.(Appendix I)

Inclusion criteria for the study involved consenting patients, symptomatic patients sent for malaria test in the laboratories, volunteers as well as patients who declined their enrolment were not involved in the study.

3.2.3 Sample Collection

3ml of peripheral blood samples were collected fron the arte-cubital vein and transferred into citrate phosphate dextrose and heparinised tubes. Thick blood film were smeared for each sample immediately after collection.

3.2.3.1 Microscopy

Preparation of Thick Blood Films

A drop of blood of each patients sample was placed at the centre of a clean microscope slide. The cover of each syringe needle was used to spread the blood drop in a circular motion to approximately a diameter of 0.4cm.The slides were allowed to air dry (Cheesbrough, 1999; Baker and Silverton, 2001)

3.2.3.2 Preparation of 5% Giemsa in Phosphate Buffer (pH 7.1)

5gram of Giemsa powder was weighed using a weighing balanace.0.41 gram of Na_2HPO_4 and 0.65 gram of KH_2PO_4 was also weighed and dissolved in 100ml of distilled water. After dissolution of the solute, 5gram of Giemsa was added to the mixture, shaken properly and allowed to stand for 24hours.

3.2.3.3 Staining of the Thick Blood Films

The dried unfixed thick blood films samples were stained using 5% Giemsa solution in phosphate buffer (pH 7.1). The slides were allowed to stand for 20 minutes after which they were washed using tap water (without streaming).After washing, the slides were kept in an upright position to air dry (Cheesbrough,1999; Baker and Silverton, 2001; Lebbad, 2004).

3.2.3.4 Examination of the Stained Thick blood films slides

A drop of oil immersion was placed on the Giemsa stained thick blood films and mounted on the microscope. This was examined using objective X100 lens after focusing.

The malaria parasites seen were identified and a grading scheme of (+) =10 parasites, (++) = 11-20, (+++) = more 30 parasites was used to establish the levels of parasitaemia.

3.2.3.5 Determination of the level of Antigenaemia in positive blood samples using Rapid Diagnostic Test Cassette

The malaria Plasmodium test cassettes was removed from their foil pouch and placed on a dry working surface. Each test cassette was labeled with the patient laboratory number. A drop of each patient blood sample was placed to the sample well on the test cassette and 3 drops of diluents added immediately. The test was allowed to run for 10 minutes. The presence of a purple color band in the result window of the test cassette served as a guide for the interpretation of the results. The presence of a purple color band on the test reaction zone and the control reaction zone in the result window implied the patients sample was positive whereas a purple color band on the control reaction zone in the result window implied the patients sample was negative and the test valid. To add, the intensity of the purple band on the test reaction zone in the result window was used to establish the level of antigenaemia. That is, light colored bands = (+); moderately intense colored bands = (++); Highly intense colored bands = (+++).This test detects Plasmodium antigens (Histidine-Rich Protein 1 and Histidine-Rich protein 2 for falciparum and the other human specie respectively).Validity of the test was considered was considered within 5-20minutes .

3.3.4 Preparation of Thin Blood films from Microscopic and Rapid Diagnostic Test Positive samples.

A drop of blood sample positive for both Standard Microscopy and Rapid Diagnostic test (RDT) were placed at 1cm away from the edge of the slide. A spreader was placed in tangentially to the blood, then allowed to spread along it, holding the slide and the spreader at a suitable angle, the spreader was pushed along the slide drawing the blood behind it until the drop has smeared. This was carried out such that the smear was thicker at the origin than at the tail end (Cheesbrough, 1999; Baker and Silverton, 2001; Lebbad, 2004).

3.3.4.1 Staining of the Thin Blood films from Microscopic and Rapid Diagnostic Test positive samples

The dried thin blood films were fixed using absolute methanol for 1 minute.5% Giemsa solution in phosphate buffer was used to stain the slides in a staining surface in the laboratory. The slides were allowed to stain for 20 minutes. The slides were later washed with tap water and allowed to air dry in an upright position.

3.3.4.2 Examination of the Stained Thin Blood films from Microscopy and Rapid diagnostic Test positive samples

A drop of oil immersion was placed on the Giemsa stained thin blood film and mounted on the microscope. The slides were examined using X100 objective lens after focusing. The examination of the malaria parasite on the slide for the speciation of the

Plasmodium specie as well as the grading scheme of (+), (++),(+++) was carried out according to Cheesbrough,1999;Baker and Silverton,2001.

3.3.4.3 Preparation of Heat inactivated pooled human serum for use in culture media

The 500cc blood bag (Type A, Rh^{+}) was hung and allowed to stand for 24 hours. The blood bag was placed in a water bath set at a temperature of $57^{o}C$. The blood bag was removed from water bag and sterilized using 70% ethanol. Using a 10ml syringe, aliquots of the serum was dispensed into 50ml falcon tubes. The serum was allowed to cool. The falcon tubes were then capped, labeled with the serum type and date, and stored in the refrigerator until use. Heat inactivation is to remove or destroy compliment factors present in the serum. (Anonymous, 2004)

3.3.4.4 Preparation of Complete Malaria Culture Medium

The complete malaria culture medium (cMCM) was prepared from a mixture of 456.5ml of RPMI 1640 liquid,5.0ml of 10mMHypoxanthine,5.0ml of 100mM l-Glutamine,12.5ml of 1M HEPES,100µl of Gentamicin sulfate and 55.0ml of pooled heat inactivated human type A serum in a sterile hood cupboard to a total volume of 534.1ml in a 1000ml volumetric flask. This was labeled with date of preparation and stored in a refrigerator. (Anonymous, 2004)

3.3.4.5 Preparation of Gradients (4 gradients) for the separation of Plasmodium falciparum mature stages using Sucrose-sorbitol density- gradient centrifugation

2 solutions were prepared i.e 50ml of solution H and 30ml of solution L respectively.

a) For Solution H:

90gram of sucrose was weighed using a weighing balance and dissolved in 100ml of distilled water (90% sucrose).5 gram of sorbitol was also weighed and dissolved in 100ml solution containing sucrose.45ml of sucrose/sorbitol mixture was added to 5ml of RPMI 1640 liquid.

b) For Solution L:

1.5 gram of sorbitol was weighed and dissolved in 27ml of distilled water.3ml of RPMI 1640 liquid was added to prepare the 30ml solution (Fernandez, 2004)

c) Preparation of the Gradients:

A 40% gradient was prepared by adding 8.3ml of solution L to 6.7ml of solution H

The 60% gradient was prepared by adding 5.0ml of solution L to 10ml of solution H

The 70% gradient was prepared by adding 3.3ml of solution L to 11.7ml of solution H

The 80% gradient was prepared by adding 2.2ml of solution L to 17.8ml of solution H.

(Fernandez, 2004)

3.3.4.6 Separation of Plasmodium falciparum mature stages in Sucrose-Sorbitol gradient

Using 4 separate 10ml syringes, 3ml, 3ml, 3ml, 4ml of each of the gradients i.e. 40%, 60%, 70%, 80% respectively was dispensed into 15ml plastic test tubes starting with the lightest solution.

The infected blood samples were washed using Tris-Hank solution and re-suspended in 1ml of solution L. a 100µl of heparin was added and the suspension passed through a 21 gauge needle 5ml syringe several times to disrupt rosettes. Each suspension of the various samples was layered on the gradients and centrifuged at 10.000 rpm for 30 minutes at 20^{o}C.After centrifugation; the supernatant at the top was removed. Using a 5ml syringe with a 21gauge needle, the mature stages were aspirated into clean 5ml test tubes from the separation pattern (debris and lysed RBC at 40% sucrose volume, mature stages at 60% and 70% sucrose volume, and uninfected and ring-infected RBC at 80% sucrose volume).2ml of Phosphate Buffered Saline (PBS) was added to the elulate and centrifuged at 3.000 rpm for 1 minutes.

(Fernandez, 2004)

3.3.4.7 Culturing of Erythrocytic Asexual Stages of Plasmodium falciparum

Human blood type O^{+} was washed several times in Tris-Hank solution to remove serum, leukocytes present. 200µl of the packed cells was added to 4ml of complete malaria culture medium in a 25ml culture flask. The elulate was added to the medium. The flask was kept in a candle jar at 37^{o} C. The cMCM was replaced daily as the level of parasitaemia increased. The culture was subcultivated by adding 200µl of O^{+} Rh^{+} ve erythrocytes and 4ml of cMCM. (Haeggstrom *et al*., 2004).

3.2.5 Estimation of Level of Parasitaemia from Culture Media

To obtain more than 10% level of parasitaemia, 50µl of culture to 4ml of cMCM for each sample according to the method of Haeggstrom and Schlichtherle,2004.

3.2.5.1 Small Scale Genomic DNA Isolation from Plasmodium falciparum

2ml of culture with 10% level of parasitaemia was placed in 500µl eppendorf tubes and centrifuged at 3.000rpm for 2 minutes. The supernatant was removed and 1ml of phosphate buffered saline was added and centrifuged at 3.000 rpm for 2 minutes. The supernatant was removed and 10µl of 5% saponin added to each tube. This was immediately centrifuged at 6.000 rpm for 5 minutes after lysis was observed. 10µl of proteinase K was added and centrifuged at 3.000 rpm for 5 minutes. 25µl of lysis buffer and 75µl of distilled water was added to the pellet. The tubes were incubated for 3 hours in a water bath at 37^{o} C with intermittent shaking by switching on the shaker. 100µl of distilled water was added to the pellet and 200µl of phenol. The pellet were mixed properly and centrifuged at 2.000 rpm for 8 minutes.200µl of chloroform was added to the supernatant and centrifuged at 2.000rpm for 8minutes.The supernatant was removed using clean micropipette with new tip into 500µl Eppendorf tubes.5µl of RNase to the mixture and incubated for 45minutes.After incubation, the tubes were centrifuged at 2000rpm for 8minutes.The supernatant were removed, air dried and reconstituted with 50µl. (Schlichtherle and Wahlgren, 2004)

3.2.5.2 Characterization of the DNA on 0.8% Aagarose

The identification of DNA was established on 0.8% agarose concentration using 5X TBE buffer as the running buffer.

3.2.5.3 Quantification of DNA Concentration

10 µl 0f the DNA sample was transferred to 900µl of TE buffer in a 1ml quartz cuvette using a 1000µl micropipette. The mixture was mixed thoroughly. The cuvette was placed into the sample well in the Eppendorf Biophotometer. The device was programmed to read the absorbance at 260nm for a double stranded DNA. The absorbance ratio at 260/280 was documented for each sample.

3.2.5.4 Nested Polymerase Chain Reaction (PCR)

A total reaction volume of 50µl per PCR reaction was used as shown in Table 3.3a below:

Table 3.1.

Ingredient	**Amount for 1 reaction**	**Amount for total reactions**
10X Buffer	5µl	
dNTPs (1.25mM)	8µl	
$MgC1_2$ (25mM)	5µl	
AmpliTaqgold	0.25µl	
CrtPl Fw	1.5µl	
CrtP2 Rv	1.5µl	
DNA Extract	5µl	
Double distilled H_20	23.75µl	
Total volume	**50µl**	

A PCR master mix was prepared using the volumes of PCR reagents listed in Table 1 excluding the volume of extracted DNA in the mix in a PCR laminar flow preparation station. 45µl of the PCR master mix was dispensed into 250µl Eppendorf tubes using a 100µl micropipette. Thereafter, 5µl of each extracted DNA samples were then being added into the 250µl Eppendorf tubes using separate 100µl micropipette tips. The tubes were then placed into the PCR machine programmed under the following conditions.

Lid temperature of 105^0C

Initial activation of 94^0C for 10minutes

Segments 1 – 94^0C for 15seconds i.e. Denaturation

Segments 2 - 56^0C for 30seconds i.e. Annealing

Extension temperature at 60^0C for 1minute.

Loop 1 for 45 cycles

With a final hold at 4^0C for 30minutes.

Table 3.2. The second nested PCR run had the following components blended in 50µl reaction volume.

Ingredient	**Amount for 1 reaction**	**Amount for total reactions plus one**
10X Buffer	5µl	
dNTPs (1.25mM)	8µl	
$MgC1_2$ (25mM)	5µl	
AmpliTaqgold	0.25µl	
CrtDl Fw	1.5µl	
CrtD2 Rv	1.5µl	
1st PCR Product	1µl	
Double distilled H_20	27.75µl	
Total volume	**50µl**	

During the second PCR, 49µl master mix was prepared as shown in Table 2 above and

dispensed into separate 250µl Eppendorf tubes. Using separate 100µl micropipette, 1µl of the first PCR product was added to 250µl Eppendorf tubes and placed in the PCR machine programmed to run under the following conditions:-

Lid temperature at 105^0C.

Initial activation at 94^0C for 10minutes

Segment 1 – 94^0C for 15 seconds. i.e. Denaturation

Segment 2 - 48^0C for 48 seconds. i.e. Annealing

Segment 3 – 65^0C for 30 seconds. i.e. Extension

Loop 2 for 30 cycles

Then final holding temperature at 4^0C for 30 minutes (Joanne, 2004)

3.2.6 Restriction Endonuclease Analysis

The restriction enzyme for the study was *Apo*I (*Athrobacter protophormiae*). Restriction enzyme analysis was carried out following the manufacturer's instruction. *ApoI* cuts a 34bp from the wild type allele but does not cut the mutant one. The positive and negative controls was however included in all steps.

To add, 1µg of the amplicon from the second nested PCR product was digested with 0.13U of *ApoI* and the corresponding enzyme buffer to 50µl reaction volume during 6 hour incubation at 50^0C in a water bath, (Joanne, 2004).

3.2.7 Agarose Gel Electrophoresis

Running buffer was prepared by mixing 40ml of 5X TBE buffer with 360m1 of distilled water. Using a top loading weighing balance, 2% of agarose was prepared (0.2g of agarose was dissolved in a mixture of 20ml 5X TBE and 80ml distilled water). This was

dissolved completely by heating in a microwave oven for 2minutes. Using a protective clothing (Kitchen Gloves), the beaker containing the gel was removed and allowed to cool to 40^0C. Using a 10µl micropippette. 0.5µl of Ethidium Bromide (EtBr) was added and mix by swirling the mixture. The glass plate of the Horizontal electrophoretic tank was adjusted from it original position in order to form a gel cast. The warm agarose solution was poured into the glass plate and immediately clamped with a 16 – tooth gel comb which formed gel slot or the sample wells into to position. **Precaution:** The warm agarose solution was gently poured into the glass plate at an estimated angle of 45^0 to prevent trapping of air bubbles. After the gel was completely set for 30minutes, at room temperature, the gel comb was removed carefully and the glass plate mounted back into it original position. The running buffer (5X TBE) was added to cover the gel to a depth approximately 1mm and allowed to soak the gel cast for 15mins. When the restriction digest was completed, 3µl of gel loading buffer (which contains 10% glycerol, 7% sucrose, and a tracking dye (0.025% bromophenol blue) was mix with 5µl of each sample with the aid of separate 100µl micropipette tips on a biofilm surface. The mixtures were then gently applied into the gel wells against DNA markers on a separate well on the gel cast. The power supply unit was switch on to run at 95V for 1 hour. After 1 hour, the power supply was switched off and the gel slab removed. The band in gel was viewed using a UV Transilluminator and photographed using a Digital camera. (Sambrook *et al*., 1989; Anonymous, 2008)

Precautions:

Ethidium Bromide (EtBr) is carcinogenic. Hand gloves were used throughout the research work. Bleach was used to decontaminate the entire gel after use

After eluting the bands on gel cast, the gel slabs was disposed off by packaging and burning using an incinerator behind the Research centre.

3.3 DATA ANALYSIS

From the photographs, the number of individual with pfcrt gene was determined presence of resistant gene was establish prevalence chi square was used to determine the association of chloroquine resistance with sex and age in the study area. This was carried out using Microsoft excel statistical software.

CHAPTER FOUR

RESULTS

This study was carried out from February 2009 to August, 2010. A total of 678 human blood samples were collected between this period to detect for human plasmodium specie using Microscopy, Rapid Diagnostic Test and PCR/RFLP. Our findings showed that, the mean percentage prevalence of malaria positive cases recorded using Microscopy and Rapid Diagnostic Test was 53.0% and 12% respectively (Table 1a). The t-test statistical analysis showed that, there was a significant difference between Microscopy and Rapid Diagnostic Test. Also, the level of sensitivity and specificity observable was 100% for both techniques.

Table 4.1: Comparison between Microscopy and Rapid Diagnostic Test

Technique	NE	NP	%Mean Prevalence	Sensitivity	Specificity
Microscopy	678	159	53	100	100
RDT	159	36	12	100	100

t-Test = 0.107 d.f =2, OR = 1.05

The prevalence of malaria parastaemia observed was higher in males than females with mean percentage prevalence values of 32.01% and 28.23% respectively. However, the student t-test statistical analysis showed that, there was no significance association between gender and malaria infection among the subjects (Table 2).

Table 4.2: Malaria parasitaemia with respect to sex

SEX	NE	NP	%Mean Prevalence
Females	425	120	28.23
Males	253	81	32.01
Total	**678**	**201**	

t-Test = 0.214 d.f =2, OR = 0.83

From the study, it was observed that the mean percentage prevalence of pregnant women who tested positive 50.00% and 26.22% for non-pregnant women. Also, our student t-test statistical analysis revealed that, there was a strong significant association between pregnancy and malaria infection (Table 3).

Table 4.3: Malaria parasitaemia with respect to Pregnancy status

Pregnancy Status	**NE**	**NP**	**%Mean Prevalence**
Pregnant Women	36	18	50.0
Non-pregnant Women	389	102	26.22
Total	**425**	**120**	

t-Test = 0.169 d.f =2

In this study, it was observed that the mean percentage prevalence of malaria parasitaemia among patient from tertiary institution was higher in males (18.57%) than in females (8.7%). The student t-test revealed that there was significant difference between malaria infection among patient from tertiary institution. (Table 4a)

In secondary institution, the mean percentage prevalence was also higher in males (22.92%) than in females (6.82%). the student t-test also revealed that, there was a significant difference between malaria infection in patient of this socio-economic class. (Table 4b).

In primary institution, the mean percentage prevalence observed was higher:- males (20.15%) than in females (8.4%). The t-test statistical analysis revealed that, there was also a significant difference between malaria infection among this social class.

Furthermore, the mean percentage prevalence among others (Traders, businessmen, drivers etc) was higher in females (17.17%) than in males (12.64%). The student t-test statistical analysis revealed that there was no significant difference between malaria infection among this social class. (Table 4d).

Table 4.4: Malaria parasitaemia with respect to Socio-Educational Status

Tertiary Institution

SEX	NE	NP	%Mean Prevalence
Female	37	12.33	8.7
Male	47	15.66	18.57

t-Test = 0.42 d.f =2, OR = 0.04

Table 4.5: Malaria parasitaemia with respect to Socio-Educational Status

Secondary Institution

SEX	NE	NP	%Mean Prevalence
Female	29	9	6.82
Male	58	19.33	22.92

t-Test = 0.26 d.f =2 , OR = 0.05

Table 4.6: Malaria parasitaemia with respect to Socio-Educational Status

Primary Institution

SEX	NE	NP	%Mean Prevalence
Female	36	12	8.4
Male	51	17	20.15

t-Test = 0.29 d.f =2

Table 4.7: Malaria parasitaemia with respect to Socio-Educational Status

Others (Traders, Businessmen, Drivers etc)

SEX	NE	NP	%Mean Prevalence
Female	73	24.33	17.17
Male	32	10.66	12.64

t-Test = 0.39 d.f =2

In this study, it was observed that the mean percentage prevalence was higher in females (7.5%) between the age group 21-25 years. In male subjects the mean percentage prevalence was also observed between he ages of 16-20 years. The total percentage prevalence among females (15.32%) and 18.40% in males. Our student t-test statistical analysis showed that, there was a significant difference between malaria infections with age. (Table 5)

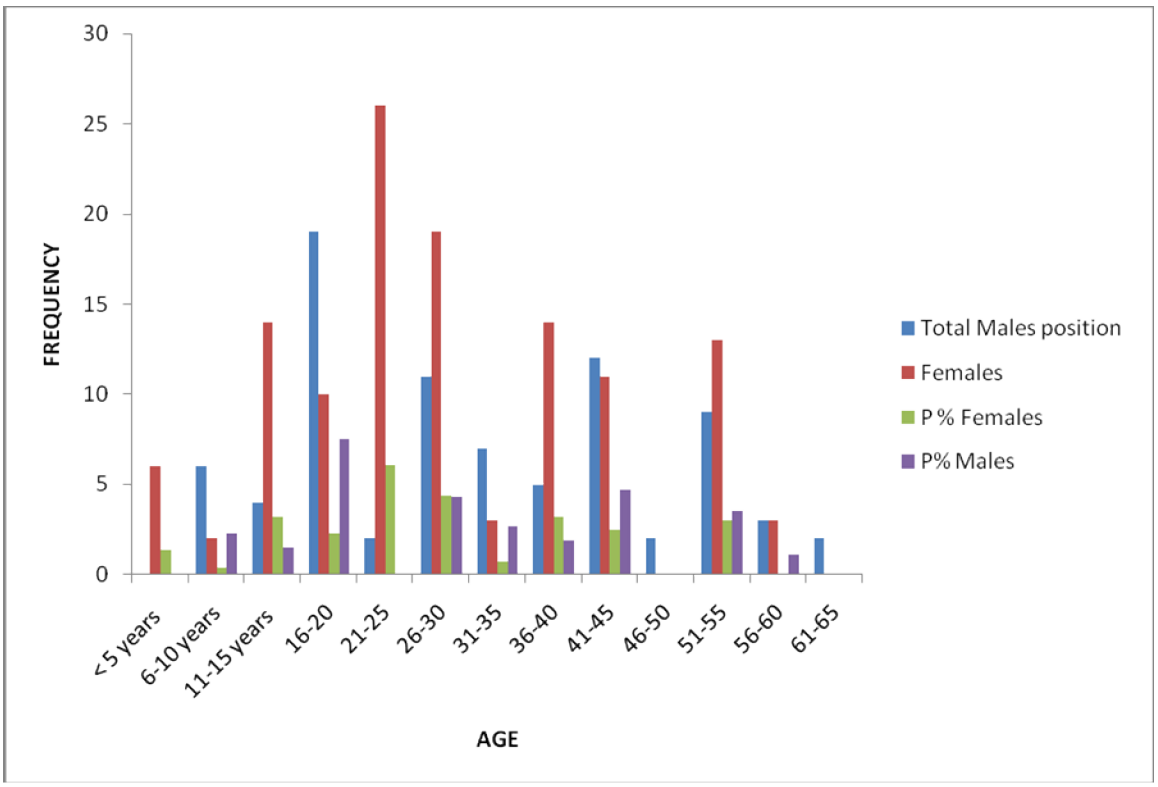


Fig 4.1: Malaria parasitaemia with respect to age

Total Mean Prevalence in females = 15.32

Total mean prevalence in males = 18.4

t- test = 0.26 d.f =12

The mode of treatment and preference for quinoline was higher in males (71.63%) than in females (54.88%).

With regards to the choice for traditional medicine as a form of therapy, the percentage prevalence was also higher in males (20.56%) than in female subject (12.03%).

Lastly the choice for other drugs such as Fansidar, Artemisinin derivatives was observed to higher in females (45.48%) than in males (24.11%). (Table 6).

Table 4.8: Mode of treatment and preference for antimalarial drugs

Quinoline	NE	NO	P%
Female	266	146	54.88
Male	141	101	71.63
Traditional Medicine			
Female	266	32	12.03
Male	141	29	20.56
Others			
Females	266	121	45.48
Male	141	34	24.11

The abundance and incidence of Plasmodium species was predominantly *Plasmodium falciparum* with a percentage prevalence of 28.93% and 18.23% for *Plasmodium malariae.* The student t-test statistical analysis revealed that there was a significant association between malaria infection and the plasmodium specie. (Table 7)

Table 4.9: Abundance/Incidence of Plasmodium species in the study area

SPECIES	NE	NP	P%
P. falciparum	159	46	28.93
P. malariae	159	29	18.23

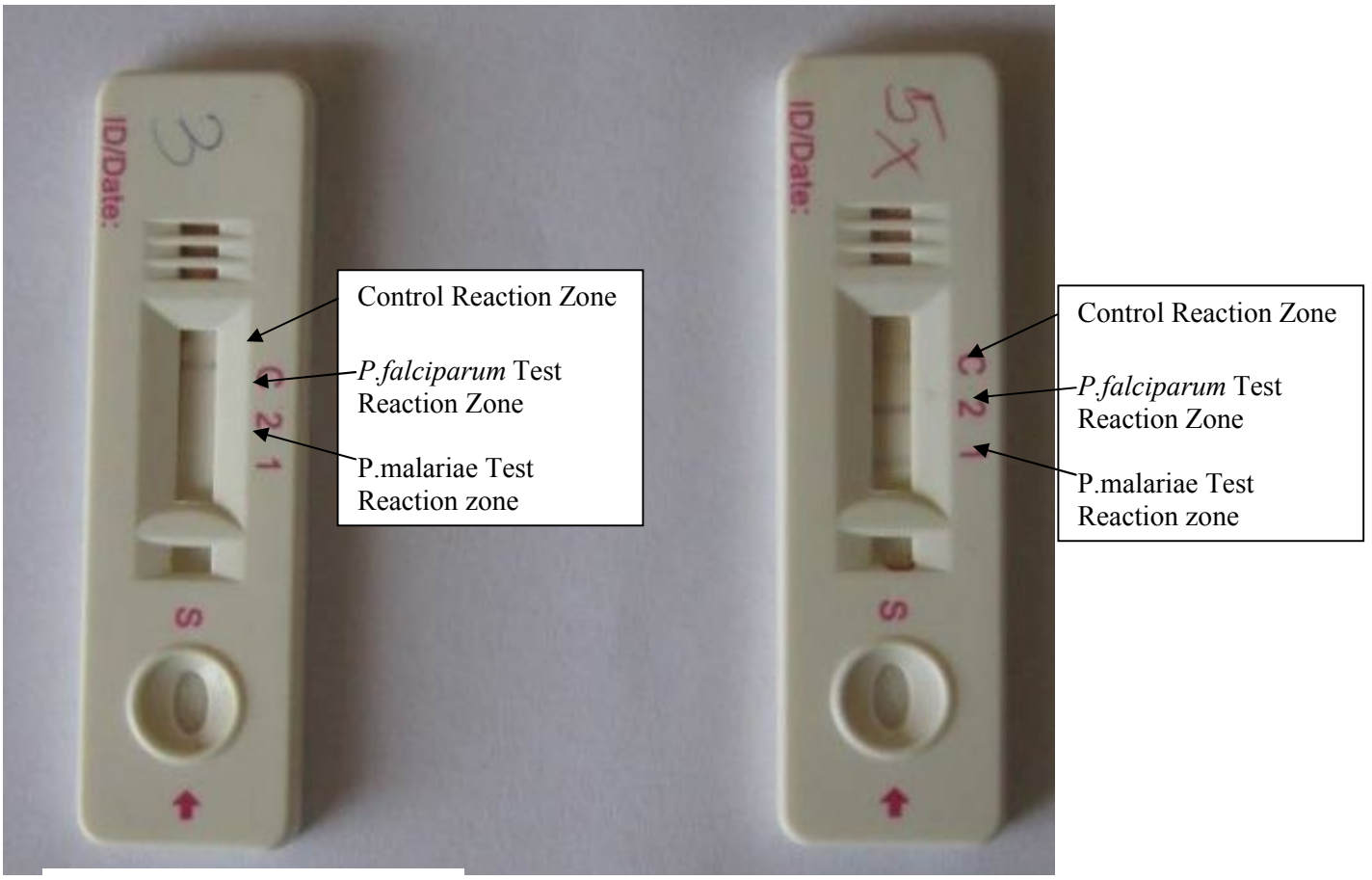
t-Test = 8.5 d.f =2

The level of parasitaemia from blood samples tested microscopically using the + (1-10 parasites), ++ (20-30 parasites), +++ (> 30 parasites) grading scheme, there was a higher prevalence in females having a + level of parasitaemia (31.9%) than in males. The ++ level of parasitaemia was also common in females (21.37%) than in males (19.34%). Also, the +++ level of parasitaemia was common only among males (14.18%) than in females (8.8%). (Table 8).

Table 4.10: Level of parasitaemia from Microscopy

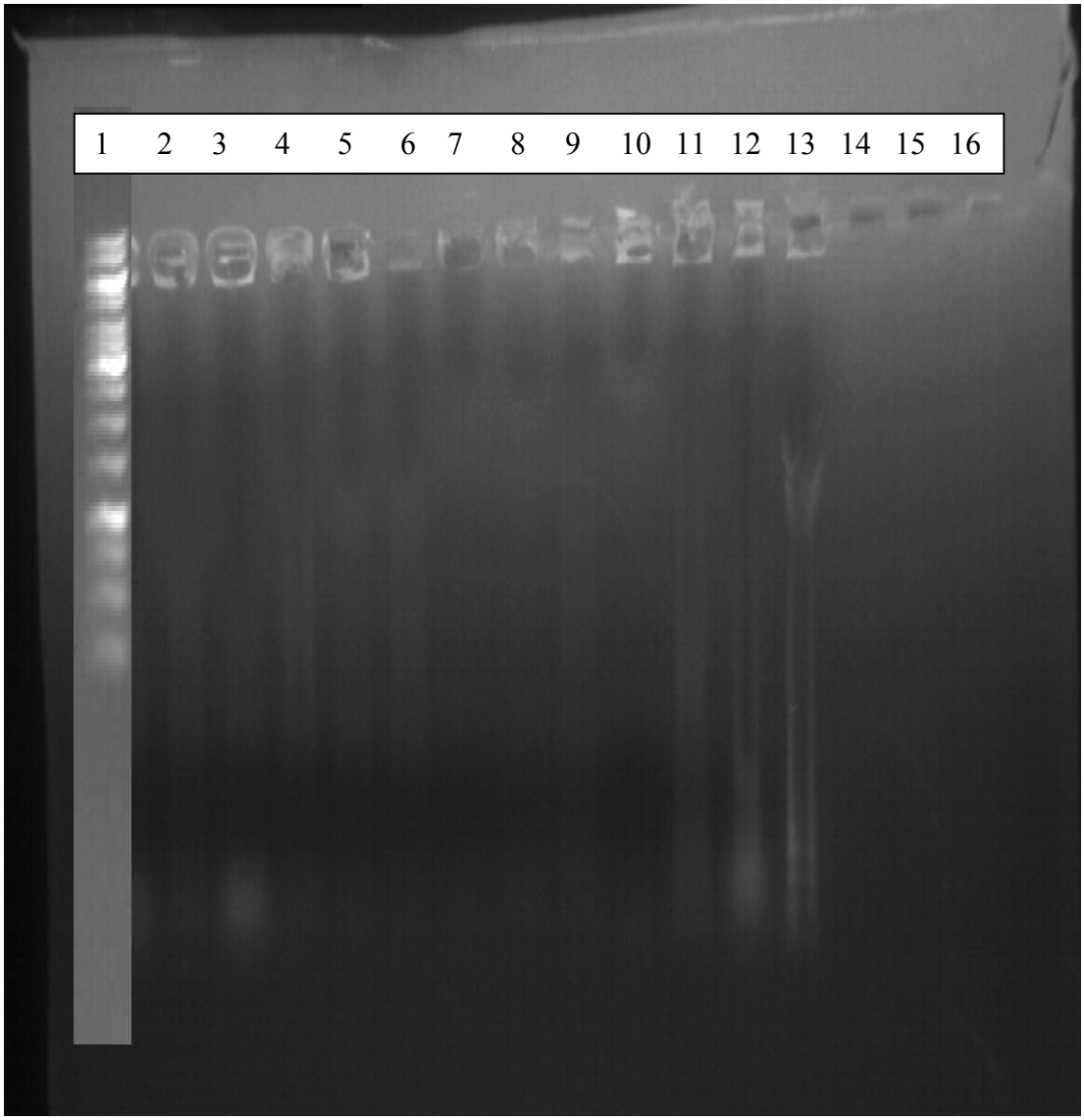
+1	NE	NP	%Mean Prevalence
Females	248	45	31.9
Males	41	35	14.11
2+			
Females	248	53	21.37
Males	148	27	19.84
3+			
Females	248	22	8.8
Males	141	20	14.18

t-Test = 0.39 d.f =2



Negative Test Cassette **Positive Test Cassette**

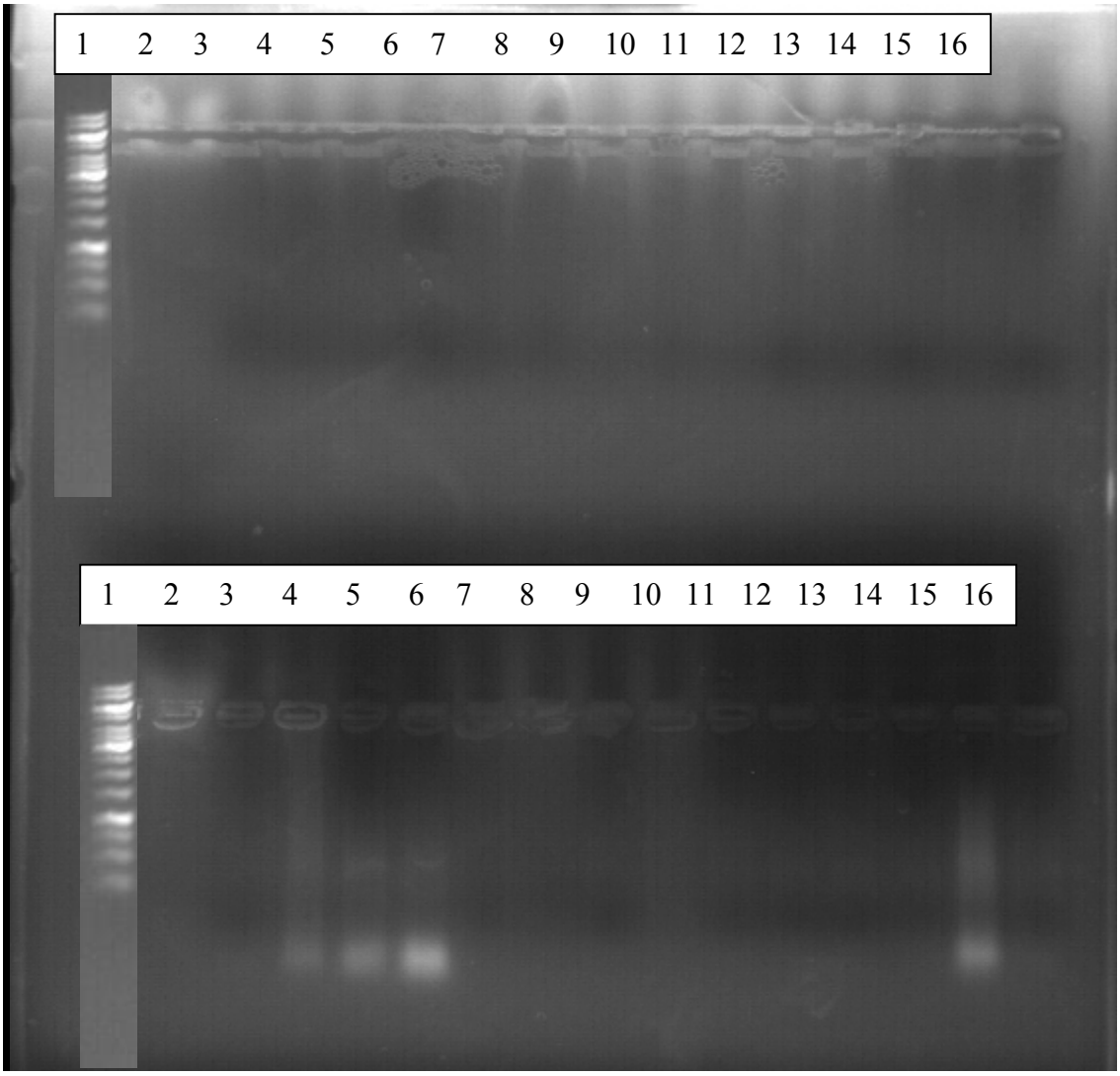
Plate I: **Test Results on Rapid Diagnostic Test Cassettes**



KEY:

1 = 1Kb DNA LADDER
2 – 16 Gel wells

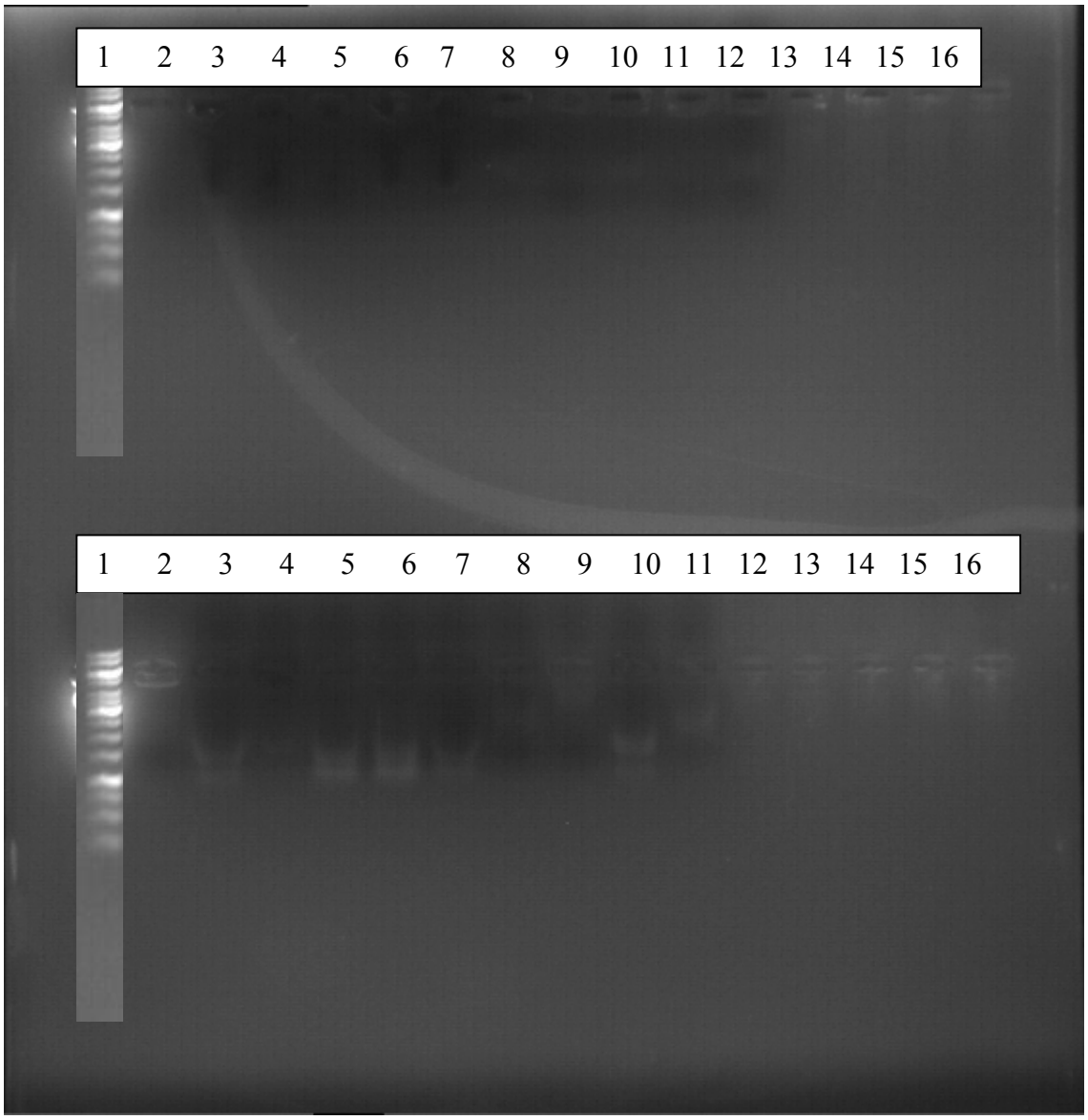
Plate II Visualisation on UV Transilluminated for purity of DNA



KEY:

1 = 1Kb DNA LADDER
2 – 16 Gel wells

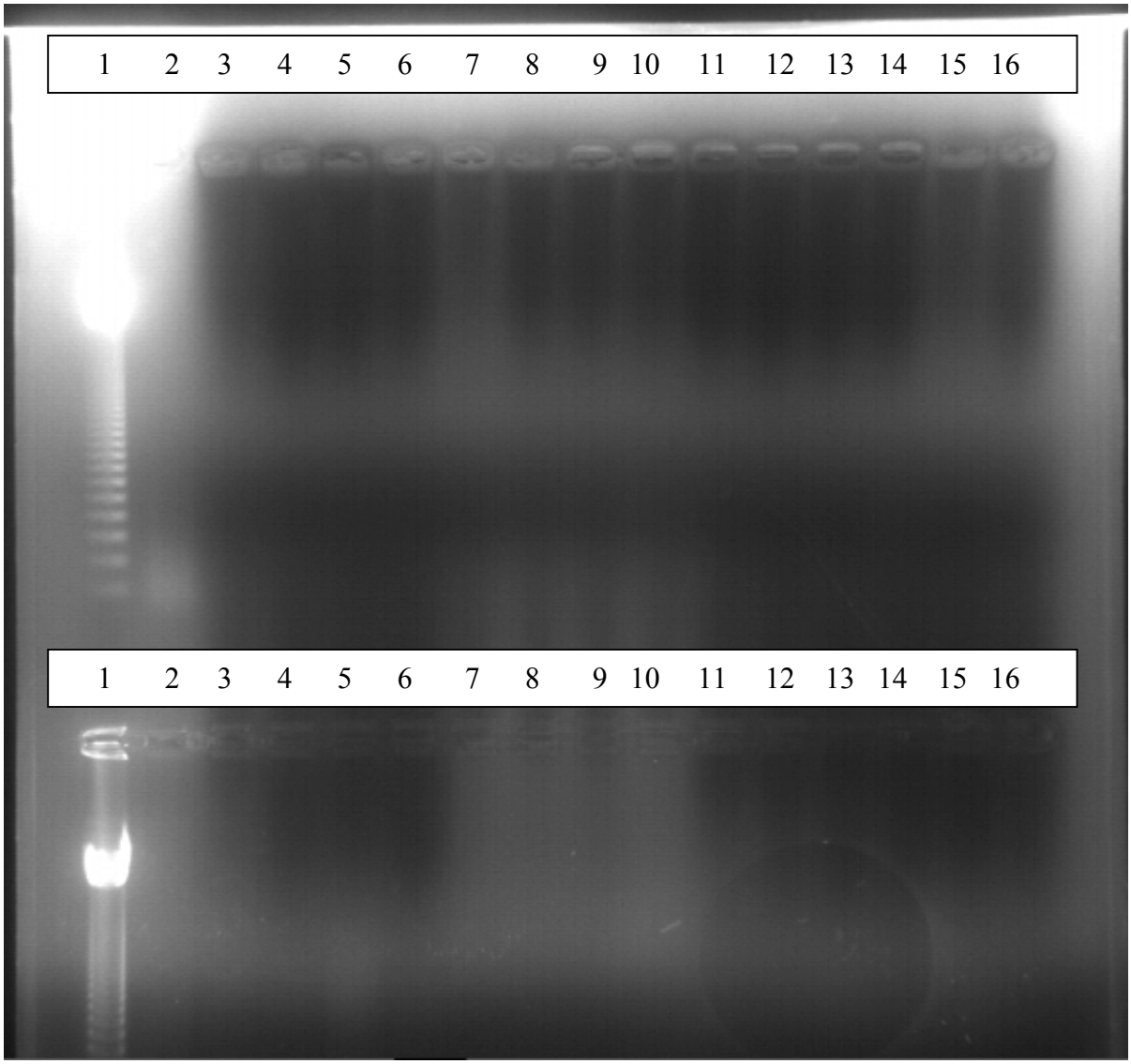
Plate III. **Nested PCR 1 products**



KEY:

1 = 1Kb DNA LADDER
2 – 16 Gel wells

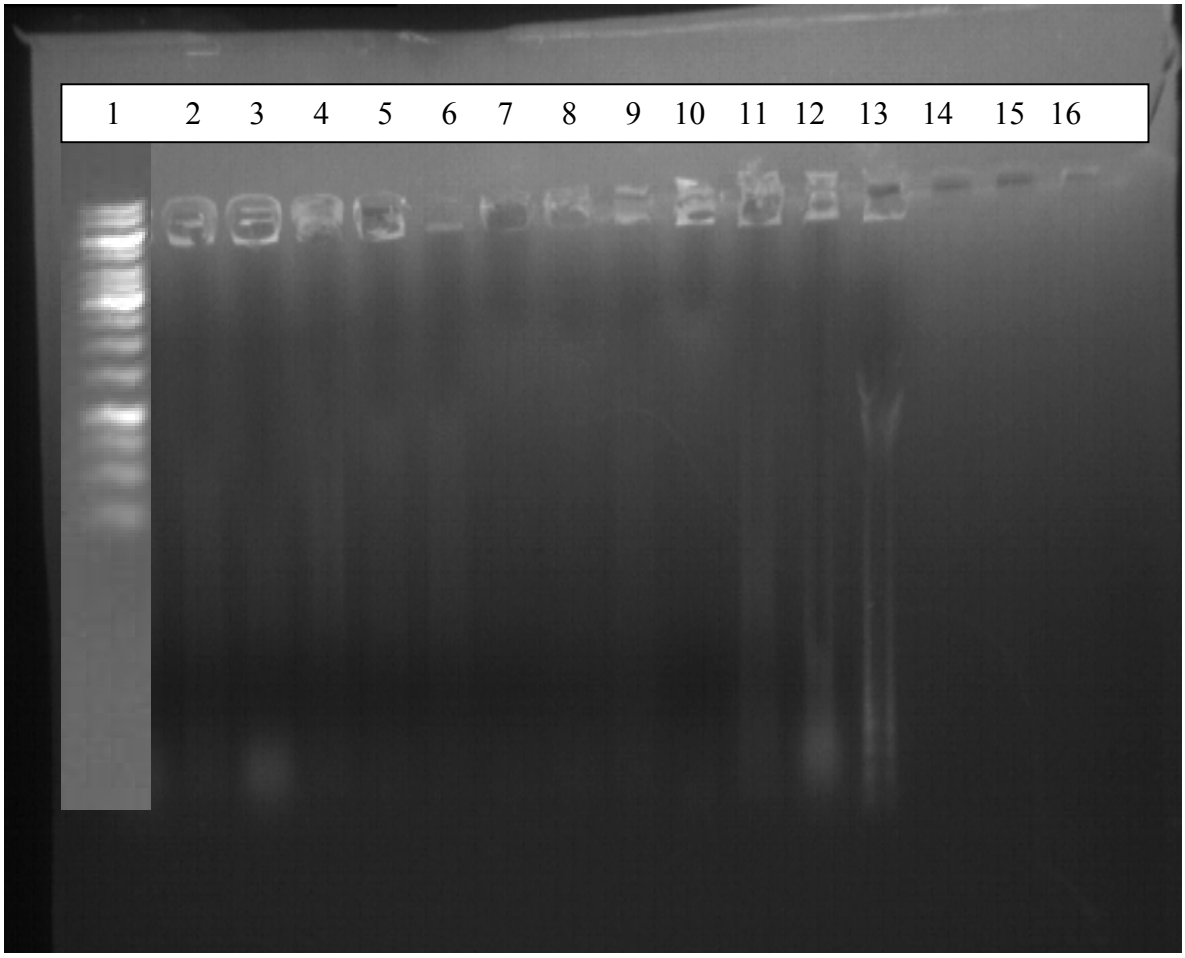
Plate IV. **Nested PCR 2 products**



KEY:

1 = 1Kb DNA LADDER
2 – 16 Gel wells

Plate V. Restriction Digest Results.



KEY:

1 = 1Kb DNA LADDER
2 – 16 Gel wells

Plate VI. Restriction Digest Results.

CHAPTER FIVE

5.0 DISCUSSION

In the study we tested blood samples from consenting patients who tested positive both for 5% Giemsa stained thick and thin blood films as well as Rapid Diagnostic Test. Result from both techniques employed were considered positive for malaria parasitaemia.

Our findings using both Microscopy and Rapid Diagnostic Test showed that, there was a higher sensitivity and specificity in the use of Rapid Diagnostic Test. Most patients confirmed positive by standard microscopy were confirmed negative by Rapid Diagnostic Test. These findings agree with reports by WHO, 2001; CDC, 2005 which showed the sensitivity of both the quantitative procedure varies with the grading scheme expertise of the personnel and poor compliance to the procedure involve in carrying out the test.

Also, our present day findings suggest from the student t- test result show that there was a significant association between malaria infection and gender in the study area but with a higher infection in males than females. This could be explained by the fact that males are more active outdoor in the evening hours than female. This findings is in conformity with reports by Kaliyaperrumal *et al*.,(2009) who suggested that outdoor activity is predominant in males than females in most developing countries due to socio cultural norms.

The percentage mean prevalence among pregnant women (50.0%) observed was high in the study area. The student t-test also suggested that there was a significant association between malaria infections with pregnancy. Guyatt, (2004) reported that malaria infection in pregnant women may be attributed to a transient depression of cell mediated immunity

that allows foetal allograft retention which interferes with resistance to various infection. Similarly , this could lead to risk of maternal anemia, delivering low birth weight babies and infant mortality generally.

It has been observed in this study from the student t-test that, there was a significant association between malaria infection and the socio-educational status of the patient in the study area. A better understanding of this may suggest that there is a linkage between malaria and poverty. Similarly, Worall *et al.,*(2003) reported that the socio-economic status of most people living in developing countries remain challenging based on how household welfare characteristics are utilized. Similarly, Holtz *et al*., (2002) suggested that SES is a complicated relationship since educational attainment is likely to have an independent bearing on an individual and access information regarding malaria prevention methods. Also, Bondi, (1991) found out that pediatric coma admission in a central Nigeria hospital consisted primarily of people with low socio economic background. Furthermore, Worall *et al*., (2003) reports show that, there is a relationship between malaria incidence or vulnerability and socio economic status.

In this study, the prevalence of malaria was common in males in the study area was between the age group 16-20years. Also a smaller percentage of children aged 6 month -7 years were encountered in both males and females in the study. This could be attributed to the facts most of the patient attending the hospitals are predominant students. Conversely, malaria infection in female in the study was between the age group 21-25years. This prevalence could be due to the fact that this study was conducted in middle of dry season and the onset of rainy season. Ukoli (1999) reports agree with these findings that, this period is marked as a period of higher transmission.

The use of anti-malarial drugs by the patient varies considerably among the types of anti-malarial drugs used and the sex in the study. A greater percentage of subject used quinolines compared to traditional herbs and other anti-malarial drugs (Fansidar, artemisinin derivatives). It was observed in this study that anti-malarial drugs are used as prophylaxis with doctor's prescription. This agrees with reports by Akanbi *et al*., (2005) whose findings also suggest that antimalarial drugs use for prophylaxis is as a result of awareness created through education on homes management and control of malaria. Also, there is need to encourage and empower researchers or scientists to identify the active ingredient in the effective local herbs for mass production. There could eventually lead to drug discovery in an effort to eliminate malaria.

In our present study *Plasimodium falciparum* constituted the most predominant species in the study (28.93%) while *Plasmodium malariae* constituted least (18.23%) Snow *et al*., 2004 reports reveals that the dominant malaria in Africa is caused by *P. falciparum* accounting for close to 100% of malaria cases. This is due to lack of Duffy receptors on the erythrocytes of Africans.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

In conclusion, this study revealed that there is a slight disparity in both the standard microscopy and Rapid Diagnostic test though microscopy is commonly employed in detection of malaria parasite, there is need for proper and trained expertise of personnel to be deployed in routine clinical examination.Again,an update in policy decision requires a consistent evidence based information that utilizes proper methodologies and sensible criteria to effect treatment as well as alternative efficacy and cost. Presumptive rule diagnosis has resulted to misdiagnosis and unnecessary treatment of patients for malaria.

From the result, better control measures should be employed through the sensitization of the masses in order to Roll Back Malaria. The control measures such as proper treatment management strategies, use of treated insecticides mosquito nets, proper environmental sanitation and hygiene will prevent infection by the parasite among different age groups, sexes, socio-economic classes etc. Where uncertainty arises in the proper clinical test or investigation, Rapid Diagnostic Test should be employed since these techniques are able to detect the malaria parasite.

Counterfeit drugs should be monitored effectively by institutions such as NAFDAC to avert the dilemma. This is due to the fact that, there is no reliable way for the doctor/physician or lay people to detect counterfeit drugs without help from a laboratory.However, these drugs are an important source of avoidable death in developing countries according to Newton *et al*.,(2006). Antimalarial drugs such as fansidar,

proguanil, mefloquine should be established concurrently to update antimalarial drug resistance in the study area.

6.2 RECOMMENDATIONS

From our present day study, we recommend that further research tools be carried out on other antimalarial drug such as sulfadoxine- pyrimethamine, proguanil, within the study area to provide a good surveillance data on antimalarial drug resistance that/which provide stakeholders, involve in malaria control programme arrive at valuable strategies and policies in curtailing drug resistance menace. Also update in surveillance data will help to improve effective control strategies that will prevent a new gene pool of the vector (*Anopheles gambiae*) thereby resulting in inefficacy of the anti malarial drugs and insecticides.

This study also recommends that patients tested negative for malaria parasitaemia by standard microcopy, who still suffer re-surgencies of malaria fever be retested using both standard microscopy and Rapid Diagnostic Test. This is to prevent misdiagnosis and unnecessary treatment for malaria in many malaria suspected subjects.

Generally, presumptive rule diagnosis should be avoided to enable malaria suspected patient to undergo laboratory test before onset of treatment for malaria. This will help prevent emergence of antimalarial drug resistance resulting due to much exposure of the parasite to the drug leading to a selection under pressure whereby the parasite mutate to circumvent therapeutic effect of the drug.

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APPENDIX I

QUESTIONNAIRE

Code Number ----------

1Age:

a) 6m-5yrs [ ] (b) 6 – 10yrs [ ] (c) 11 – 15yrs [ ] (d) 16 – 20yrs [ ]

(e) 21 – 25yrs [ ] (f) 26 -30yrs [ ] (g) 31-35yrs [ ] (h) 36 – 40yrs [ ]

(i) 41 – 45yrs [ ] (j) 46 -50yrs [ ] (k) 51 -55yrs [ ] (l) 56 -60 [ ]

(m) 61-65yrs [ ]

(2) Gender

(a) Male [ ] (b) Female [ ] bii) Pregnant females ( )

(3) Educational Status

(a) Tertiary [ ] (b) Secondary [ ] (c) Primary [ ] (d) Others [ ]

(4) Type of antimalarial treatment used

(a) Quinine and its derivative [ ] (b) Traditional medicine [ ] (c)Others

(Artemisinin derivative, Halfan, Fansidar etc) [ ]

(5) Level of Parasitaemia

(a) + [ ] (b) ++ [ ] (c) +++ [ ]

(6) Test result from RDT cassettes

a) *P. falciparum* ( ) b) *P. malariae and others* ( )

APPENDIX II

CONSENT FORM

Studies on malaria prevalence, drug treatment regimes and intensity of chloroquine resistant *Plasmodium. falciparum* in zaria.

This study is to evaluate the efficiency reproducibility and repeatability of both the standard microscopy and rapid diagnostic test cassettes used in the diagnosis of malaria. The positive malaria samples will then be used to also evaluate the status of the drug to assess its therapeutic effect as well as providing useful information on drug surveillance using the modern techniques to establish resistance of antimalarial drugs.

Your cooperation and participation will facilitate the validity test used and efficacy of the drug in relation to the age, gender, treatment with antimalarial drugs etc.

Again, you will be expected to donate 3ml of your blood. During this process, you will experience a little pain due to venous puncture that wane in a 5minutes and usually no treatment is required. If you are in accordance with this study, please sign below

Name and address of donor: ----------

Name of researcher: ----------

APPENDIX VIII

Table 1a: Comparison between Microscopy and Rapid Diagnostic Test

Technique	NE	NP	%Mean Prevalence	Sensitivity	Specificity
Microscopy	678	159	53	100	100
RDT	159	36	12	100	100

t-Test = 0.107 d.f =2, OR = 1.05

APPENDIX IV

Table 2: Malaria parasitaemia with respect to sex

SEX	NE	NP	%Mean Prevalence
Females	425	120	28.23
Males	253	81	32.01
Total	**678**	**201**	

t-Test = 0.214 d.f =2, OR = 0.83

APPENDIX V

Table 8: Level of parasitaemia from Microscopy

+1	NE	NP	%Mean Prevalence
Females	248	45	31.9
Males	41	35	14.11
2+			
Females	248	53	21.37
Males	148	27	19.84
3+			
Females	248	22	8.8
Males	141	20	14.18

t-Test = 0.39 d.f =2