Prevalence of molecular markers associated with chloroquine, sulphadoxine-pyrimethamine and lumefantrine resistance following the deployment of artemether-lumefantrine as first-line treatment for uncomplicated *falciparum* malaria in Gaza Province, Mozambique

By

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As the candidate's supervisor I have/have not approved this dissertation for submission.

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I, Juanita Chewparsad, declare that

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PREFACE

The experimental work described in this thesis was carried out in the molecular research laboratories of the Malaria Unit, Medical Research Council, Durban. This work was carried out under the supervision of Dr. Jaishree Raman and Professor J. P. Dean Goldring. The studies represent original work by the author and have not otherwise been submitted in any other form to another University. Where use has been made of the work of others it is duly acknowledged in the text.

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ABSTRACT

Antimalarial drug resistance is a major contributing factor to the sustained malaria burden in sub-Saharan Africa. Under extreme drug pressure, drug resistant parasites have a selective fitness advantage over wild-type parasites. However this selective advantage is negated once the drug pressure is removed. Over the past decade, malaria treatment policy in Mozambique has changed at least twice. In 2006 the monotherapy chloroquine (CQ) was replaced by artemisinin-based combination therapy artesunate plus sulphadoxine-pyrimethamine (SP) which in turn was replaced by artemether-lumefantrine in 2008. This study investigates the effect these changes in drug pressure had on the prevalence of molecular markers associated with CQ, SP and lumefantrine resistance in Gaza Province, Mozambique. Finger prick filter paper blood samples were collected from malaria rapid diagnostic test positive children at 20 sentinel sites during annual cross-sectional malaria prevalence surveys in 2010 and 2011. Chelex extracted parasite DNA confirmed as being *P. falciparum* positive using quantitative and nested PCR methods was subjected to mutational analysis using nested PCR and RFLP protocols to determine the prevalence of molecular markers associated with SP,CQ and lumefantrine resistance. Over the study period, the prevalence of SP resistance markers were nearing fixation, while all samples analysed had a single copy of the *pfmdr1* gene. Despite the reduction of SP drug pressure since 2008, the prevalence of SP resistance markers were still high suggesting that SP pressure still exists in the region, raising concerns over the efficacy of intermittent preventative treatment using SP. This study supports the change from artesunate plus SP to artemether-lumefantrine in Gaza Province The prevalence of CQ resistance markers in the *pfcrt* gene increased over the study period whilst a decrease was observed in the *pfmdr1* gene. These results suggest that CQ could still be in use in Gaza Province probably due to a lack of ACTs and availability of CQ. Whilst markers of artemisinin resistance have yet to be identified, the selection of the *pfmdr1* 86N allele, indicative of lumefantrine resistance, suggests that the *pfmdr1* gene is under selection because of CQ withdrawal and raises concern for the continued use of the drug combination in the region. Further studies, using other markers of artemether-lumefantrine resistance are required. The *pfmdr1* 86Y marker was found to be associated with the *pfcrt* CVIET haplotype and the SP quintuple marker prevalence with residence influencing this association. This study contributes to the body of knowledge regarding drug resistance markers in Gaza Province which could be used to inform drug policy in the future.

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ABBREVIATIONS

ºC	Degrees Celsius
µl	Microliters
µM	Micromoles
ACT	Artemisinin-based combination therapies
bp	Base pairs
BSA	Bovine serum albumin
CI	Confidence interval
CQ	Chloroquine
CVMNK	*Plasmodium falciparum pfcrt* codons 72-76 wildtype haplotype
CVIET	*Plasmodium falciparum pfcrt* codons 72-76 wildtype haplotype
dH_2O	Distilled water
DHF	Dihydrofolate
dhfr	*Plasmodium falciparum* dihydrofolate reductase gene
dhps	*Plasmodium falciparum* dihydropteroate synthetase gene
dNTPs	Deoxynucleotide triphosphates
DV	Digestive vacuole
EtBr	Ethidium bromide
GLURP	Glutamate-rich protein
HRP-2	Histidine-rich protein 2
IDC	Intra-erythrocytic development cycle
IPTp	Intermittent preventive treatment for pregnant women
LSDI	Lubombo Spatial Development Initiative
$MgCl_2$	Magnesium chloride
MOI	Multiplicity of infection
msp-1	Merozoite surface protein 1
msp-2	Merozoite surface protein 2
OR	Odds ratio
PABA	*p*-amino benzoic acid
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pfcrt	*Plasmodium falciparum* chloroquine resistance transporter gene
PfCRT	*Plasmodium falciparum* chloroquine resistance transporter protein

pfmdr1	*Plasmodium falciparum* multidrug resistant gene 1
q-PCR	Quantitative polymerase chain reaction
RBC	Red blood cell
RDT	Rapid diagnostic test
rRNA	Ribosomal ribose nucleic acid
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphisms
SP	Sulphadoxine-pyrimethamine
TAE	Tris acetate EDTA
THF	Tetrahydrofolate
WHO	World Health Organization

Amino acid abbreviations:

A	Asparagine
C	Cysteine
E	Glutamic acid
G	Glycine
I	Isoleucine
K	Lysine
L	Leucine
N	Asparagine
S	Serine
R	Arginine
Y	Tyrosine

CHAPTER 1: Introduction and Literature Review

1.1. General introduction

Malaria, a parasitic vector-borne disease, remains a serious health problem in the developing world (Stratton *et al*., 2008) (Figure 1.1). Several causes for the continuous presence of the disease have been suggested and include population movement into malarious regions, increases in agricultural development, weakening of public health systems, global warming and the spread of insecticide and antimalarial drug resistance (Sachs and Malaney, 2002). Limiting the emergence and spread of antimalarial drug resistance is one of the biggest challenges facing public health officials attempting to control malaria (Menard *et al.*, 2006).

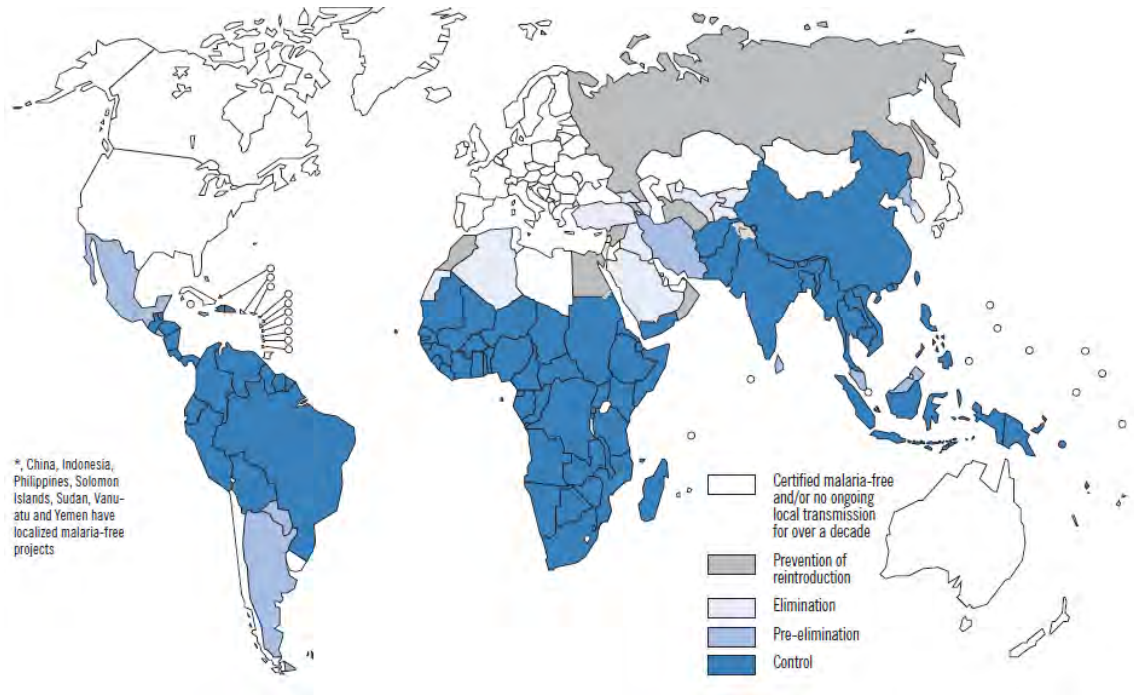


Figure 1.1: The global distribution of malaria showing malaria free countries and malaria endemic countries in various phases of elimination and control in 2008 (World Health Organization, 2009a).

1.2. The global burden of malaria

Malaria is endemic to 106 countries with approximately 3.3 billion people at risk of infection annually (World Health Organization, 2011a). Globally, it was estimated the disease resulted in 216 million clinical malaria episodes and over 655 000 deaths in 2010 (World Health Organization,

2011a). In sub-Saharan Africa, 47 countries are endemic for malaria (World Health Organization, 2010a). Approximately 81% of malaria cases and 91% of deaths due to malaria occur in Africa; with the highest burden of morbidity and mortality reported in children below the age of five years and pregnant women (World Health Organization, 2011a).

Malaria is not only a severe health burden; it also imposes a heavy economic and social burden on endemic countries. Economic activity is compromised by the premature loss of life, time lost due to illness, medical care and prevention and treatment of the disease (Barofsky *et al*., 2011; Gollin and Zimmerman, 2007). Social costs include the consequences of poor health and impaired cognitive development on the quality of life in severe malaria cases (Barofsky *et al*., 2011; Fernando *et al*., 2010; Gollin and Zimmerman, 2007). Sachs and Malaney (2002) suggested that there is a striking correlation between malaria and poverty. Their research showed that malaria contributed to poverty by impeding economic growth (Sachs and Malaney, 2002). Poverty was shown to negatively impact malaria control efforts due to their associated costs (Sachs and Malaney, 2002). Annually, Africa has a loss in income of twelve billion US dollars due to malaria (Vitoria *et al*., 2009).

1.3. Malaria disease

1.3.1. The mosquito vector

Malaria is transmitted by female mosquitoes belonging to the genus *Anopheles.* Of the 400 species of *Anopheles* mosquitoes present worldwide, 60 transmit malaria (Breman, 2001). In Africa, there are three main malaria vectors: *Anopheles gambiae sensu stricto* and *An. arabiensis* which both belong to the *An. gambiae* complex, and *An. funestus* which belongs to the *An. funestus* group (Cohuet *et al*., 2003; Hougard *et al*., 2002; Spillings *et al*., 2009). The efficiency of these mosquitoes as vectors of the disease is due to their anthropophily, endophily and long life (Greenwood and Mutabingwa, 2002). Mosquito behaviour is greatly influenced by seasonal fluctuations in climate, hence malaria transmission by *An. gambiae* mosquitoes is seasonal, whilst with *An. funestus,* malaria transmission is perennial (Gillies and Coetzee, 1987).

1.3.2. The *Plasmodium* parasite

Malaria is a parasitic disease caused by the infection of erythrocytes with intracellular protozoa belonging to the genus *Plasmodium* (World Health Organization, 2009a). Members of this genus are capable of causing malaria in a variety of different organisms including mammals, birds and reptiles. Historically only four *Plasmodium* species, *viz. Plasmodium falciparum*, *P. vivax*, *P. malariae* and *P. ovale* have been associated with human malaria (Gregson and Plowe, 2005; World

Health Organization, 2009b). However, recent reports from Southeast Asia suggest that *P. knowlesi*, the causative agent in simian malaria, is also capable of human infection (Cox-Singh and Singh, 2008; White, 2008) although this is primarily a zoonosis (Lee *et al*., 2011). *Plasmodium falciparum* is considered to be the most lethal human parasite in addition to being the most prevalent (Byakika-Kibwika *et al*., 2010). *Plasmodium falciparum* is most often associated with severe malaria because of its a) indiscriminate infection of both young and old erythrocytes and b) ability to avoid spleen clearance by sequestering in the brain and other vital organs (Dhangadamajhi *et al*., 2010; Heddini, 2002).

1.3.3. Life-cycle of *Plasmodium falciparum*

The *P. falciparum* life-cycle is complex requiring both a human and a mosquito host. In brief, the cycle begins when malaria sporozoites are transferred from the salivary glands of an infected female Anopheline mosquito into the dermis of a human whilst the mosquito is feeding (Gregson and Plowe, 2005; Yamauchi *et al*., 2007) (Figure 1.2, Box 1). The sporozoites can move around in the skin until they enter the blood stream, a process that can occur immediately or extend for hours after the initial bite (Sinnis and Zavala, 2008; Yamauchi *et al*., 2007). While the duration of sporozoite infectivity is debated (reviewed by Yamauchi *et al*., 2007), it is thought that sporozoites enter the liver and invade hepatocytes within half an hour after infection (Gregson and Plowe, 2005; Matuschewski, 2006). In the hepatocytes, the sporozoites develop into schizonts (Figure 1.2, Box 2) and over a 6 to 10 day period in the *P. falciparum* life-cycle, they undergo repeated schizogony (multiplication by mitosis) forming merozoites which are discharged from the hepatocytes into the blood stream (Barnes and White, 2005; Gregson and Plowe, 2005; Figure 1.2, Box 3). This begins the *P. falciparum* asexual intra-erythrocytic developmental cycle, associated with the clinical symptoms and pathology of the disease, where the merozoites invade erythrocytes, multiplying to form progeny merozoites (Bozdech *et al*., 2003; Gregson and Plowe, 2005; Mauritz *et al*., 2010; Yamauchi *et al*., 2007). After a period of 48 hours, characteristic of the *P. falciparum* parasite, and after progressing through the ring, trophozoite and schizont stages of maturation; the progeny merozoites are released from the infected erythrocyte. These merozoites either invade uninfected erythrocytes, continuing the intra-erythrocytic cycle or differentiate into gametocytes, the sexual forms of the parasite responsible for the transmission of malaria (Buffet *et al*., 2011; Gregson and Plowe, 2005; Figure 1.2, Box 4). When a mosquito feeds on the infected human host, if both male and female gametocytes are taken with the blood-meal, fertilization may occur to form a zygote in the mosquito midgut (Barnes and White, 2005; Dhangadamajhi *et al*., 2010; Gregson and Plowe, 2005; Matuschewski, 2006; Figure 1.2, Box 5). The zygote then matures into an infective ookinete which migrates through the midgut wall, attaching itself to the outer gut wall (Dhangadamajhi *et al*., 2010). The ookinete then matures into an oocyst which forms (over a period

of 10-22 days) and releases sporozoites (Figure 1.2, Box 6) that migrate to the salivary glands of the mosquito, via the body cavity and other organs, awaiting transmission to a new host (Dhangadamajhi *et al*., 2010; Gregson and Plowe, 2005; Matuschewski, 2006; Tilley *et al*., 2011).

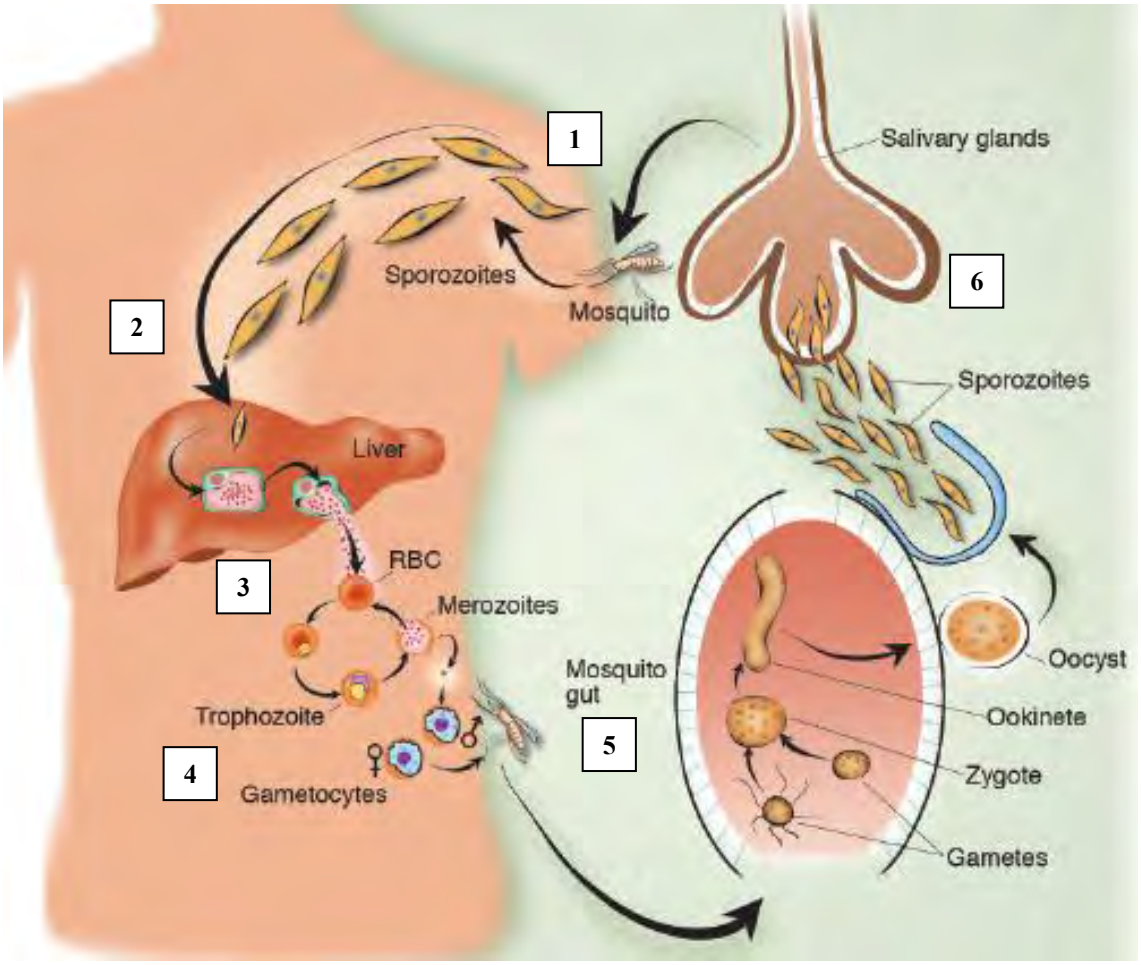


Figure 1.2: The life-cycle of a *P. falciparum* parasite (Adapted from White, 2004).

1.3.4. Clinical Symptoms of Malaria

The erythrocytic stage of the parasite life-cycle is responsible for clinical symptoms of malaria. Infection with the *P. falciparum* parasite can result in either an asymptomatic, uncomplicated (mild) or complicated (leading to severe) malaria infection (Laishram *et al*., 2012). Asymptomatic malaria does not manifest clinically whilst uncomplicated malaria symptoms are non-specific manifesting as fever, chills, sweats, headaches, joint aches, fatigue and abdominal discomfort (Bell and Winstanley, 2004; Idro *et al*., 2005; Laishram *et al*., 2012). Severe malaria is defined by the presence of any of the following symptoms: coma (cerebral malaria), convulsions, malarial anaemia, haemoglobinuria, hypoglycaemia, metabolic acidosis, acute pulmonary oedema, acute

renal failure, jaundice, circulatory collapse, hyperparasitaemia or spontaneous bleeding (Laishram *et al*., 2012).

If untreated, an uncomplicated *P. falciparum* malaria infection can rapidly progress to complicated malaria as the parasites multiply in the body. Under increased parasite loads and with the maturation of the asexual *P. falciparum* parasites, infected red blood cells express the *P. falciparum* erythrocyte membrane protein 1, a ligand that enables adhesion to the endothelial cells lining blood vessels (Heddini, 2002; Hughes *et al*., 2010; Pettersson *et al*., 2005). This is termed cytoadherence which allows for the sequestration and attachment of parasitized blood cells in organs such as the lungs, brain and adipose tissue (David *et al*., 1983; Franke-Fayard *et al*., 2010; Hughes *et al*., 2010; Pettersson *et al*., 2005). Severe malaria is associated with high risks of neurological impairment and patients that survive severe forms of the disease can experience long term disability (Fernando *et al*., 2010; Winstanley *et al.*, 2002). The majority of untreated severe malaria cases result in death (World Health Organization, 2009b).

The severity of an infection can differ depending on individual characteristics such as age and degree of malaria specific partial immunity (premunition) (Dondorp *et al*., 2008; Olliaro, 2008; White and Pongtavornpinyo, 2003). Immunity reduces the symptoms of the disease and can lead to asymptomatic malaria infections where patients do not exhibit acute clinical signs of malaria (Bell and Winstanley, 2004; Bereczky *et al*., 2007; Idro *et al*., 2005). The level of transmission intensity in a region influences the development of immunity. In high malaria transmission regions individuals are more likely to be infected repeatedly and develop some degree of acquired immunity (Bereczky *et al*., 2007; Idro *et al*., 2005; White and Pongtavornpinyo, 2003). As a result, asymptomatic infections usually occur in children over five years of age and adults whilst infants living in these areas are at a higher risk of developing severe malaria (Bell and Winstanley, 2004; Bereczky *et al*., 2007; Idro *et al*., 2005; White and Pongtavornpinyo, 2003). In low transmission regions, immunity is less likely to develop because of the limited exposure to infection, therefore individuals of all ages are non or semi-immune and any initial malaria infection can easily progress to severe malaria infection if left untreated (Breman, 2001; Laishram *et al*., 2012; Winstanley *et al*., 2002). Pregnant women and children below the age of five, irrespective of transmission intensity, are at higher risk of progressing to severe forms of the disease than other groups. Children have not had sufficient exposure to infection to acquire immunity, whilst pregnant women have compromised immune systems (Idro *et al*., 2005).

1.4. Malaria Control

Various tools have been used to control malaria including insecticide treated bed nets, indoor residual spraying, combination drug therapy and intermittent preventive treatment (Greenwood and Mutabingwa, 2002; Steketee and Campbell, 2010). Steketee and Campbell (2010) report that whilst each control intervention may be effective in its own right, it is a combination of these control measures that provide the best control for the disease. These control methods, and a commitment to proper management of control interventions, are required in order to ensure that malaria control is as effective as possible (Najera *et al*., 2011; Steketee and Campbell, 2010).

1.4.1. Malaria vaccine

Despite advances in research and technology, a successful malaria vaccine has yet to be developed. There are, however, numerous candidate vaccines that are at various stages of development which are outlined in the World Health Organization's (WHO) malaria vaccine rainbow tables (Available at http://www.who.int/vaccine_research/links/Rainbow/en/index.htm. for full tables of vaccines at pre-clinical and clinical stages, inactive or discontinued vaccines and funders). These tables provide a summary of the malaria vaccine projects.

Malaria candidate vaccines differ in their mode of protection and targets in the parasite's life-cycle *viz.* pre-erythrocytic, intra-erythrocytic and sexual stages (O'Meara *et al*., 2007). Vaccines that target the pre-erythrocytic stage are currently the leading malaria candidate vaccines (Schwartz *et al*., 2012). These vaccine antigens may be the targets of immune responses that would either prevent sporozoite invasion of hepatocytes or kill infected hepatocytes (Schwartz *et al*., 2012). If effective, this type of vaccine could inactivate the parasite in the liver which would enable sterile immunity and may lead to the prevention of malaria (Schwartz *et al*., 2012). GlaxoSmithKline Vaccines and PATH Malaria Vaccine Initiative have developed a potential vaccine, RTS, S AS01 which targets the pre-erythrocytic stage of the *P. falciparum* life-cycle (Breman and Plowe, 2009). RTS, S AS01 is an antigen-adjuvant combination consisting of sequences of the circumsporozoite protein and the hepatitis B surface antigen with AS01, an immunostimulant adjuvant (Hill, 2011; http://www.malariavaccine.org/rd-vaccine-candidates.php). The rationale is that the vaccine would elicit both humoral and cell-mediated immune responses that will neutralize and aid the removal of the parasites soon after infection (http://www.malariavaccine.org/rd-vaccine-candidates.php). The vaccine has progressed through phase I and II clinical trials in numerous African countries and is now in phase III trials at 11 sites in seven African countries (Garcia-Basteiro *et al*., 2012). The phase I and II trial results show that RTS, S AS01E can reduce the incidence of all episodes of clinical malaria by 55% in children aged 5-17 months and preliminary phase III trial results confirm these results have an efficacy of 34.9% (Garcia-Basteiro *et al*., 2012; Hill, 2011; Schwartz *et al*., 2012).

1.4.2. Vector control

Long-lasting insecticide treated bed nets and indoor residual spraying are two broadly applied interventions targeting the mosquito vector. Both interventions aim to shorten the life-span of endophilic malaria vectors thereby restricting onward transmission of the parasite by reducing human-vector contact (Lengeler and Sharp, 2003). In sub-Saharan Africa approximately 73% of the 800 million people at risk received insecticide treated bed nets by 2010, with 96% of owners claiming to use it often (World Health Organization, 2011a). Indoor residual spraying on the other hand protected 11% of the population at risk in 2010 (World Health Organization, 2011a). Indoor residual spraying and long-lasting insecticide treated bed nets are useful for long term protection from mosquitoes because of their estimated effective insecticide life-span ranging from six months to three years (Lengeler and Sharp, 2003; World Health Organization, 2010a).

1.4.3. Parasite control by case detection and treatment

Effective control of *P. falciparum* requires correct parasitological diagnosis of the disease. A traditional method of parasite identification is microscopy, which is the gold standard for malaria detection (World Health Organization, 2010a). It is not always feasible to use microscopy in field settings as proper microscopic examination takes time and requires good quality equipment and reagents, suitable microscope operating conditions, trained microscopists and maintenance of the microscopes that may prove to be costly (Boonma *et al*., 2007; Greenwood *et al*., 2005; Hashizume *et al*., 2006).

1.4.3.1. Case detection using rapid diagnostic tests

Rapid diagnostic tests (RDTs) provide an alternative to microscopy in the detection of malaria infection (Greenwood *et al*., 2005). These devices use immunochromatography and detect specific antigens produced by malaria parasites in the blood of infected individuals, the presence of which is signified by a colour change on nitrocellulose strips (Moody, 2002; UNICEF, 2007). Commercially available RDTs detect three main groups of antigens, *Plasmodium* aldolase, *Plasmodium* lactate dehydrogenase and *P. falciparum* specific histidine-rich protein 2 (HRP-2) (Moody, 2002; UNICEF, 2007). *Plasmodium* aldolase and *Plasmodium* lactate dehydrogenase are both found in the glycolytic pathway of the parasite whilst HRP-2 is present in the cytosol and on the membrane surface of *P. falciparum* infected red blood cells (Moody, 2002; UNICEF, 2007). The aldolase detecting RDT is pan-specific whilst lactate dehydrogenase detecting RDTs are currently available as *P. falciparum* specific, *P. vivax* specific and pan-specific variants (Moody,

2002; UNICEF, 2007). The HRP-2 detecting RDT specifically identifies *P. falciparum* infections (Baker *et al*., 2010; Hendriksen *et al.*, 2011; Moody, 2002).

Rapid diagnostic tests should ideally 1) provide results that are as accurate as those derived from microscopy performed in field conditions; 2) detect parasitaemia at 100 parasites/µl of blood with 100% sensitivity (to ensure detection of true malaria cases); and 3) have 95% specificity (to avoid false positive results) (Chinkhumba *et al*., 2010; Moody, 2002). These specifications are difficult to achieve and provide a challenge to manufacturers and scientists alike. Therefore RDTs that are commercially available detect less than 100 parasites per/µl and generally have a 90% sensitivity and specificity (Marquart *et al*., 2012). The sensitivity and specificity of RDTs are however influenced by storage and transport conditions (UNICEF, 2007) and are more likely to give false results when these conditions are not met. However, RDTs are better suited to field conditions than microscopy because results are available within 15 to 20 minutes, they are transported easily and require moderate training to operate and interpret results (Kyabayinze *et al*., 2010; Moody, 2002).

Histidine-rich protein 2 RDTs were used to detect malaria infection in this field study as these RDTs are specific to *P. falciparum*; have greater sensitivity in environments with variable climates; are more temperature-stable than other RDTs and are advantageous in terms of stability, cost and format (Moody, 2002; UNICEF, 2007).

1.4.3.2. Antimalarial drug treatment

Successful parasite control can be sustained by proper antimalarial therapies. Antimalarial drugs are classified based on the status of infection, parasite life-cycle stage they are active against, molecular targets and drug half-lives (World Health Organization, 2010b). Chemoprophylaxis does not affect sporozoites and is advised for at risk individuals, pregnant women, infants and non-immune individuals travelling to malaria endemic areas (Corachan, 1997; Greenwood, 2010). There are two types of chemoprophylactic agents: a) suppressive agents which affect the erythrocytic stages and prevent clinical disease and b) causal agents which affect liver forms and prevent release into the blood (Corachan, 1997). Ideally chemoprophylaxis should prevent malaria morbidity and mortality while being safe to use, well tolerated and cost effective (Fernando *et al*., 2011). Prophylactic drugs will not be the same in different malaria settings because of differences in the local drug resistance mutants, cost effectiveness and public acceptance of the drug in each area (Fernando *et al*., 2011). The WHO does however recommend mefloquine (Lariam or Mephaquine), atovaquone/proguanil (Malarone) and doxycycline for chemoprophylaxis (Senn *et al*., 2007). Sulphadoxine-pyrimethamine (SP) is also used as a form of chemoprophylaxis in intermittent preventive treatment in pregnant women and infants (Odhiambo *et al*., 2010). Intermittent preventive treatment is the administration of therapeutic doses of an antimalarial drug

regardless of a patient's infection status at specific time points as an effort to control malaria in pregnant women and infants in areas where efficacy of that drug is not completely compromised (Greenwood, 2010).

The progression of malaria infections to severe forms of the disease could result in death if not treated promptly with the correct drugs. The statuses of infection such as uncomplicated, complicated and severe malaria need to be properly identified before treatment begins to ensure that the correct drugs are being administered to the infected individual. Antimalarial drugs for the treatment of severe malaria are aimed at preventing death and need to be safe and fast-acting (Cramer *et al*., 2011). Parenteral administration of artemether (intramuscular), quinine (intravenous and intramuscular) and artesunate (intravenous and intramuscular) are used to treat severe malaria (Dondorp *et al*., 2010). Compared to quinine, artesunate injections reduce mortality rates by 22.5% (Dondorp *et al*., 2010) and are considered to be the superior treatment for severe malaria (World Health Organization, 2010b).

Antimalarial drugs for the treatment of uncomplicated malaria infection are aimed primarily at curing the infection and eliminating the parasites from the body so preventing progression to severe malaria and death (White, 2002). A secondary aim is to prevent transmission (White, 2002). Drugs that have been used for the treatment of uncomplicated falciparum malaria in Gaza Province, Mozambique include amodiaquine, artesunate, chloroquine, sulphadoxine-pyrimethamine and artemether-lumefantrine.

Due to there being many stages of the *P. falciparum* life-cycle, different antimalarial drugs target different life-cycle stages (World Health Organization, 2010b) (Figure 1.3). Tissue schizonticides target the pre-erythrocytic cycle preventing the infection of erythrocytes (Olliaro, 2001). Most antimalarial drugs are blood schizonticides and act on the asexual intra-erythrocytic developmental cycle; their primary target appears to be the parasite digestive vacuole (Olliaro, 2001). Blood schizonticides kill asexual parasites (Olliaro, 2001). Such antimalarial drugs include the quinolines, folate inhibitors and artemisinins (Olliaro, 2001). Gametocytocides kill gametes thus reducing transmission (White, 1999; White, 2008). Thus far, only the artemisinins and 8-aminoquinolines are reported to have a gametocytocidal effect (Olliaro, 2001).

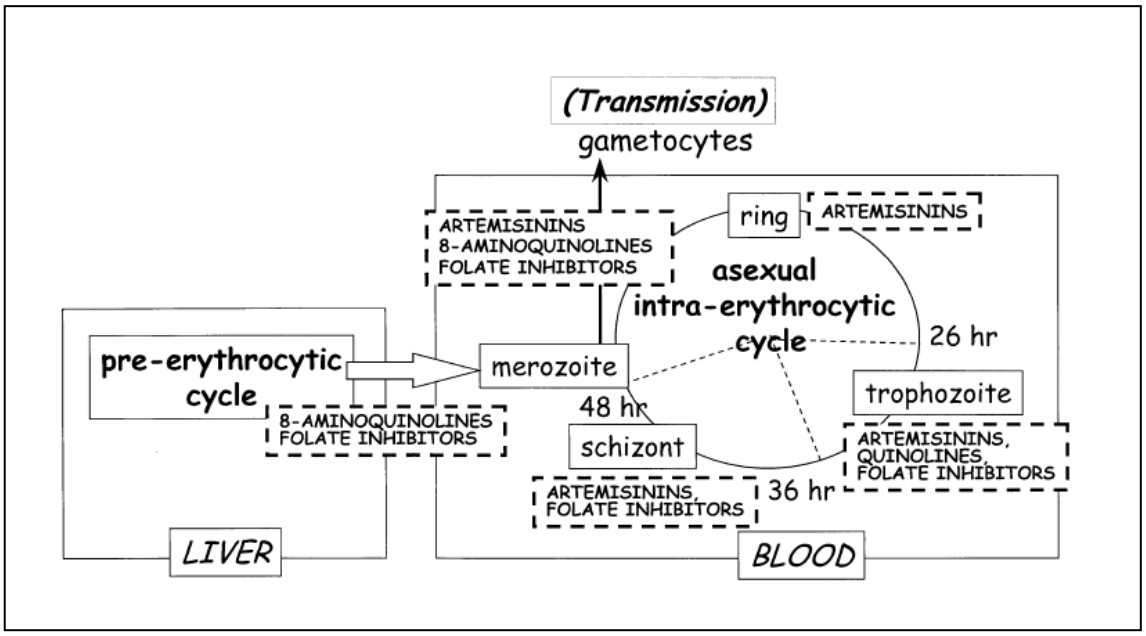


Figure 1.3: Antimalarial drugs of different classes target different stages of the malaria infection in humans (Olliaro, 2001).

Quinolines

Quinoline containing drugs are subdivided into the 4-aminoquinolines and aryl amino alcohol drug classes. These drugs act on intra-erythrocytic parasites and are therefore blood schizonticides (Bakshi *et al*., 2000). Drugs belonging to the aryl amino alcohols include quinine, halofantrine, mefloquine, lumefantrine and drugs that belong to the 4-aminoquinolines include amodiaquine, pyronaridine and chloroquine (World Health Organization, 2009b). Of interest here are lumefantrine and chloroquine as these are used in the study area.

Lumefantrine is responsible for removing the residual parasite load, when used in combination with a drug with a shorter half-life, and has a half-life of 3-6 days (Byakika-Kibwika *et al*., 2010). It prevents haem detoxification in blood schizonts and has little effect on either tissue schizonts or gametocytes (Byakika-Kibwika *et al*., 2010; Fanello *et al*., 2007). Chloroquine was one of the most widely used antimalarials since the 1940s as it was cheap, rapidly cured malaria symptoms and could be safely administered to young children and pregnant women (Burgess *et al*., 2010; Kremsner and Krishna, 2004) with a half-life of 1-2 months (Olliaro, 2001).

The mechanisms of action of quinolines have been widely debated, though a widely accepted hypothesis is that this class of drugs targets the parasite by inhibiting haem detoxification and disposal (Bakar *et al*., 2010; Olliaro, 2001). During their growth, the ring-stage parasites endocytose the cytoplasm of the red blood cell and accumulate the remnants in their digestive

vacuole (Bakar *et al*., 2010; Bray *et al*., 2005) (Figure 1.4). In the digestive vacuole, haemoglobin is digested into ferriprotoporphyrin IX/ haem (Bray *et al*., 2005; Lehane and Kirk, 2008). Due to haem being toxic to the parasite, it is polymerized into an inert crystalline substance, haemozoin (Bray *et al*., 1998; Bray *et al*., 2005). There are little data on the mode of action of lumefantrine, however its antimalarial activity correlates positively with that of other quinoline drugs, suggesting a similar mode of action (White *et al*., 1999). It is proposed that lumefantrine possibly acts by chemically inhibiting haem polymerization however further studies are required to strengthen this hypothesis (White *et al*., 1999). The mode of action of chloroquine has been studied extensively with various hypotheses suggested though consensus of a sole mode of action has not yet been reached. Chloroquine accumulates in the parasites digestive vacuole (Olliaro, 2001). Chloroquine is a weak base; at a neutral pH, it can freely diffuse through membranes whilst at acidic pH, it becomes trapped inside the digestive vacuole (Olliaro, 2001). Proposed mechanisms of haem disposal by chloroquine include the inhibition of haemoglobin degradation or haemozoin formation by increasing the pH of the digestive vacuole (Olliaro, 2001); inhibition of the peroxidative destruction of haem which is toxic to the parasite (Loria *et al*., 1999); and inhibition of the crystallization of haem into haemozoin within the digestive vacuole thereby killing the parasite (Bray *et al*., 1998; Bray *et al*., 2005; Duraisingh and Cowman, 2005; Wellems and Plowe, 2001). The chloroquine resistance transporter, which confers drug resistance to chloroquine as discussed later, is thought to play a role in inhibiting some of these processes.

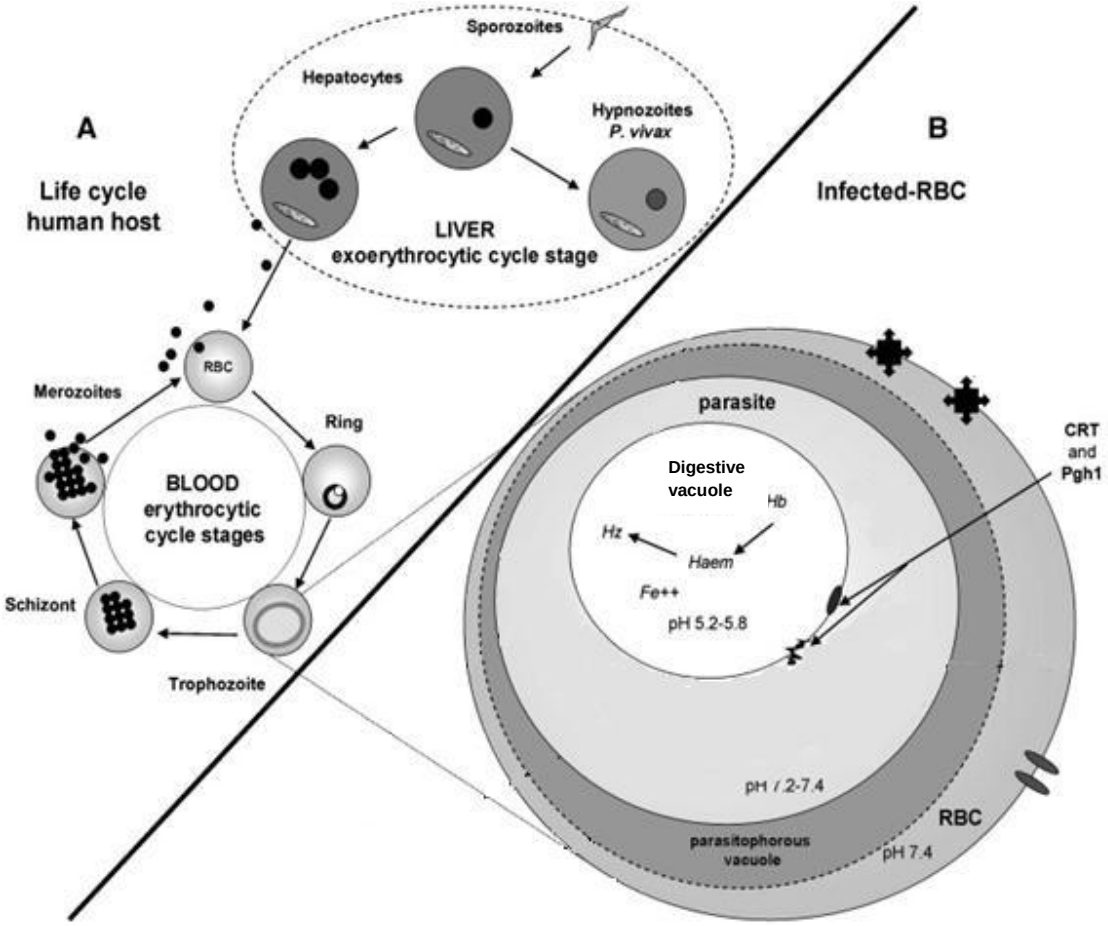


Figure 1.4: Life-cycle stage targets of chloroquine in the parasite digestive vacuole during the asexual cycle (Santos-Magalhaes and Mosqueira, 2009). RBC: red blood cell; CRT: *P. falciparum* chloroquine resistance transporter; Pgh1: *P. falciparum* p-glycoprotein homologue 1.

Folate inhibitors

The folate inhibitors are blood schizonticides that act on the folate biosynthesis pathway of the intra-erythrocytic cycle (Olliaro, 2001). Folates are composed of three building blocks: a pteridine ring, para-aminobenzoic acid and glutamic acid (Olliaro, 2001). Folate inhibitors compete with or inhibit these substrates in the folate pathway resulting in reduced protein and DNA synthesis (Morgan *et al.*, 2011; Olliaro, 2001) and include drugs such as proguanil, sulphalene, sulphone, dapsone, sulphadoxine and pyrimethamine (Hyde, 2002; World Health Organization, 2010b). Sulphadoxine and pyrimethamine are of interest in this study and will be discussed further.

Sulphadoxine, a structural analogue of the substrate *para*-aminobenzoic acid, inhibits dihydropteroate synthetase (*dhps*) activity thereby decreasing the synthesis of dihydropteroate and consequently dihydrofolate (Nzila, 2006) (Figure 1.5). Dihydropteroate synthetase catalyses the condensation of hydroxymethyldihydropterin-pyrophosphate and para-aminobenzoic acid to yield

dihydropteroate and pyrophosphate (Gregson and Plowe, 2005; Morgan *et al*., 2011; Nzila, 2006). Pyrimethamine mimics the pteridine ring of and competes with dihydrofolate to inhibit the active site of *dhfr* (Chookajorn and Kumpornsin, 2011) (Figure 1.5). Dihydrofolate reductase (*dhfr*) regulates the reduction of dihydrofolate to tetrahydrofolate (Nzila, 2006; Peterson *et al*., 1990).

Sulphadoxine-pyrimethamine (SP) is distributed as a synergistic combination drug because the inhibition of *dhps* by sulphadoxine decreases the synthesis of dihydrofolate, the substrate of *dhfr* thereby increasing the activity of the *dhfr* inhibitor pyrimethamine (Nzila, 2006). Sulphadoxine-pyrimethamine was a widely used antimalarial drug combination as it was both safe and easy to use (McCollum *et al*., 2008). Both sulphadoxine and pyrimethamine are slowly eliminated from the body (elimination half-lives of 4-9 days and 4 days respectively) exposing the parasite to its antimalarial effects for fairly long periods (World Health Organization, 2009b).

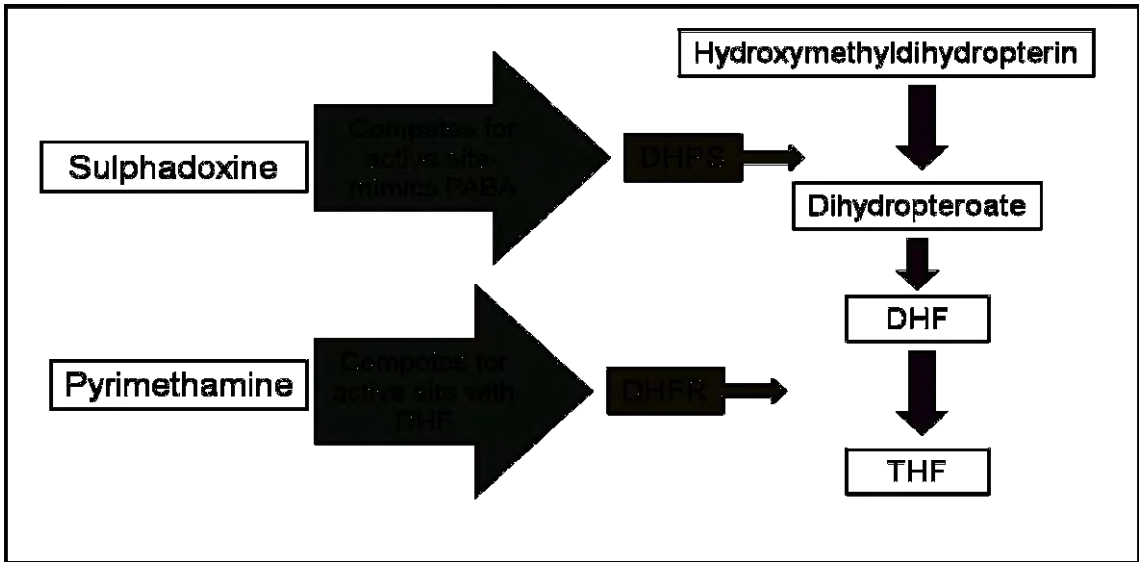


Figure 1.5: Mode of action of sulphadoxine and pyrimethamine (Adapted from Olliaro, 2001). PABA: para-aminobenzoic acid; DHPS: *dihydropteroate synthetase*; DHF: dihydrofolate; DHFR: *dihydrofolate reductase*; THF: tetrahydrofolate.

Artemisinins

Artemisinins are recommended by the WHO for treatment of uncomplicated malaria infections because they: a) have rapid cure rates due to efficient clearing of the asexual parasite load; b) have high gametocytocidal activity which reduces transmission rates and c) are quickly eliminated reducing the risk of the development of resistance (Chotivanich *et al*., 2000; Eastman and Fidock, 2009; Meshnick, 2002; White, 2004). Artesunate and artemether are two artemisinin derivatives currently available and commonly used in artemisinin-based combination therapies (ACTs). They both form the biologically active metabolite dihydroartemisinin *in vivo,* which is water soluble and rapidly absorbed by the body (Davis, 2004). Both have short elimination half-lives (determined by

dihydroartemisinin elimination) of 45 minutes for artesunate and 1-3 hours for artemether (Byakika-Kibwika *et al*., 2010; Krishna *et al*., 2004). Artemisinin toxicity has seldom been recorded and the drug is safe to use in children although, of those cases reported, treatment with artemisinin derivatives coincided with neurological problems in humans and neurotoxicity in animals (Krishna *et al*., 2004; Meshnick, 2002).

The antimalarial function of artemisinins is attributed by an intramolecular peroxide bridge situated in the sesquiterpene lactone backbone structure of the drug (Li and Zhou, 2010; Wang *et al*., 2010). The mode of action of artemisinins is debatable (Ding *et al*., 2011) though different biological models have been suggested. One model suggests that artemisinins bind and inhibit the calcium dependent endoplasmic reticulum-resident ATPase (PfATP6) (Li and Zhou, 2010; O'Brien *et al*., 2011) however, a study showed that this model is unlikely given the inability of artemisinin to bind to PfATP6 (Arnou *et al*., 2011). Another model suggested that artemisinins containing the peroxide bond may interact with the mitochondrial electron transport chain of the parasite and generate radical oxygen species which could disrupt normal mitochondrial function and lead to cell death, though more supporting evidence for this model is required (Li and Zhou, 2010; Wang *et al*., 2010). Yet another model suggested that artemisinins act inside parasite digestive vacuoles where they are activated by haem and interfere with haem detoxification, though stronger evidence of this association is required (Eastman and Fidock, 2009; Klonis *et al*., 2011; Li and Zhou, 2010; O'Neill *et al*., 2010).

As a consequence of widespread resistance to commonly used monotherapies, the WHO recommended that all malaria endemic countries use combination therapies to treat uncomplicated malaria (Dondorp *et al*., 2009; Ogbonna and Uneke, 2008; World Health Organization, 2010b). It was hypothesized that using two or more drugs with different modes of action together would improve drug efficacy without increasing toxicity, and reduce the chances of antimalarial resistance emerging and spreading (Humphreys *et al*., 2007; White, 1999; White, 2004).

Currently, artemisinin-based combination therapies (ACTs) are the only antimalarials that offer high levels of efficacy and tolerability (World Health Organization, 2010b). There are five ACTs approved by the WHO for treatment of uncomplicated *falciparum* malaria; the two most commonly used in Africa are artesunate plus sulphadoxine-pyrimethamine and artemether-lumefantrine (Enosse *et al*., 2008).

Artesunate plus sulphadoxine-pyrimethamine is a WHO recommended ACT, though resistance to sulphadoxine-pyrimethamine has severely hindered the efficacy of this combination (Raman *et al*., 2010). Artemether-lumefantrine is a synergistic combination of artemether and lumefantrine

(Eastman and Fidock, 2009). Artemether and lumefantrine have different modes of action yet both are effective against the asexual intra-erythrocytic developmental cycle (Byakika-Kibwika *et al*., 2010). Artemether-lumefantrine as a combination is relativity safe and well tolerated with mild adverse events affecting the gastrointestinal tract, dermatological and central nervous systems provided it is administered properly (Bakshi *et al*., 2000; Byakika-Kibwika *et al*., 2010).

1.5. Antimalarial drug resistance

Antimalarial drug resistance is defined as the ability of *Plasmodium* parasites to remain viable following exposure to drug concentrations normally considered lethal (Hastings, 2011). Crucial to the control of malaria is knowledge of the development and spread of drug resistance which influences policy to ensure that antimalarial drugs available in any given area are always effective.

1.5.1. Development of resistance

Although resistance can emerge via a number of different mechanisms, antimalarial drug resistance has a genetic basis, with mutations (single or multiple) in the parasite genome and/or variations in gene copy numbers reducing the parasites susceptibility to antimalarials (Gregson and Plowe, 2005; White and Pongtavornpinyo, 2003). These genetic events may affect the drug accumulating in the parasite or the affinity to its target (White, 1999; White, 2004). Some say that mutations occurred *de novo* whilst others say that mutations occurred through gene flow (Anderson and Roper, 2005; Anderson *et al*., 2011). There are two processes central to the emergence and spread of resistance (Dondorp *et al*., 2011; White and Pongtavornpinyo, 2003). These are resistance generated by mutations and spread of mutations within and between parasite populations by selection, which confers survival advantage in the presence of drugs (Anderson and Roper, 2005; Barnes and White, 2005; White, 1998; White, 2004; White and Pongtavornpinyo, 2003).

1.5.2. Mechanisms of drug resistance

1.5.2.1. Chloroquine: *Plasmodium falciparum* chloroquine resistance transporter

The exact molecular mechanisms of chloroquine resistance are still in debate. Two genes associated with chloroquine resistance are the *P. falciparum* multidrug resistance gene 1(*pfmdr1*) and the *P. falciparum* chloroquine resistance gene (*pfcrt*) (Burgess *et al*., 2010; Lehane and Kirk, 2008; Severini *et al*., 2006; Sharma, 2005). Whilst there is evidence that mutations in the *pfmdr1* gene may modulate levels of chloroquine resistance, there is a strong link between mutations in the *pfcrt* gene, specifically the mutation at codon 76 (lysine to threonine), and chloroquine treatment failure (Durand *et al*., 2001; Martin *et al*., 2009; Severini *et al*., 2006; Su *et al*., 1997). All alleles

conferring chloroquine resistance have the 76T *pfcrt* mutation with alleles in Asia and Africa having additional 7-8 amino acid changes (Anderson and Roper, 2005). The *pfcrt* gene, located on chromosome seven, encodes the *P. falciparum* chloroquine resistance transporter (PfCRT) located on the parasites digestive vacuole membrane (Burgess *et al*., 2010; Su *et al*., 1997) (Figure 1.6). Mutant PfCRT (containing the 76T mutation) has been shown to mediate the efflux of chloroquine from the digestive vacuole (Burgess *et al*., 2010; Kirk, 2004; Lehane and Kirk, 2008; Valderramos and Fidock, 2006). The *pfcrt* mutant 76T allele is well established across the African continent (Plowe, 2009).

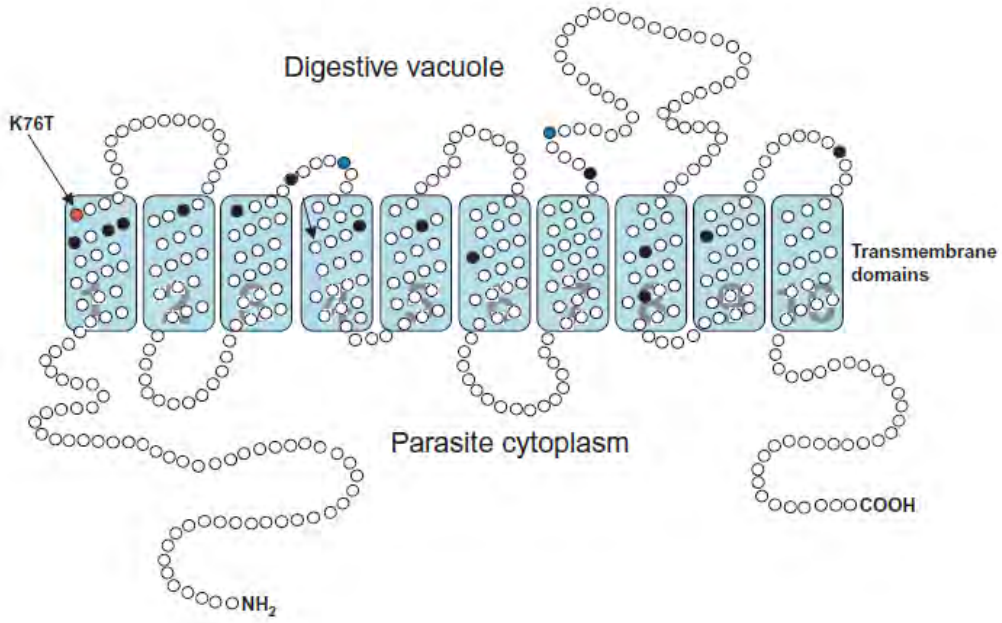


Figure 1.6: Structure of the *P. falciparum* chloroquine (CQ) resistance transporter showing the key 76T and other mutations (red and black filled circles) in the *P. falciparum* CQ resistance transporter gene (Bray *et al*., 2005).

1.5.2.2. Pyrimethamine: *Plasmodium falciparum dihydrofolate reductase*

The *dhfr* enzyme is encoded by a single-copy gene on chromosome four in the *P. falciparum* genome (Gregson and Plowe, 2005). Pyrimethamine resistance is conferred by mutations at codons 51, 59, 108 and 164 in the *dhfr* gene which change the *dhfr* active site (Alker *et al*., 2005; Basco *et al*., 2000; Plowe *et al.*, 1997). A serine to asparagine amino acid change at codon 108 (S108N) is usually the first mutation to emerge under sustained sulphadoxine-pyrimethamine drug pressure (Alker *et al*., 2005). Although almost all pyrimethamine resistant parasites carry this mutation, its presence alone is not a reliable predictor of *in vivo* pyrimethamine resistance (Alifrangis *et al*., 2003; Sharma, 2005). The presence of two additional mutations at codons 51 (asparagine to isoleucine; N51I) and 59 (cysteine to arginine; C59R), collectively known as the *dhfr* triple

mutation, provides high levels of pyrimethamine resistance (Alifrangis *et al*., 2003; Bwijo *et al*., 2003). An additional mutation at codon 164 (isoleucine to leucine; I164L), to form a *dhfr* quadruple mutation, confers an even greater level of pyrimethamine resistance (Hyde, 2008; Plowe, 2009). Parasites with the quadruple mutation have up to 600 times lower pyrimethamine binding affinities compared to wildtype *dhfr* and seven times lower than the *dhfr* triple mutation (Hyde, 2008). Mutant *dhfr* is widespread in Africa (Sridaran *et al*., 2010) and has been reported at high levels in Mozambique (Raman *et al*., 2010; Raman *et al*., 2011).

1.5.2.3. Sulphadoxine: *Plasmodium falciparum* dihydropteroate synthetase

The *dhps* gene is located on chromosome eight of the *P. falciparum* genome (Triglia and Cowman, 1994). A number of mutations in the *dhps* gene at codons 436, 437, 540, 581 and 613, thought to affect the enzymes active site, have been linked with sulphadoxine resistance (Basco *et al*., 2000; Alker *et al*., 2005). Two amino acid changes at codons 437 (alanine to glycine; A437G) and 540 (lysine to glutamic acid; K540E), known as the *dhps* double mutation (Anderson and Roper, 2005; Sharma, 2005), are strong predictors of sulphadoxine resistance throughout most of East and southern Africa (Pearce *et al*., 2009). The *dhps* 581 mutation (alanine to glycine; A581G), which is thought to confer higher levels of sulphadoxine resistance, has been reported in West and Central Africa (Pearce *et al*., 2009).

1.5.2.4. *Plasmodium falciparum* multi-drug resistance gene 1

The *P. falciparum* multi-drug resistance gene 1 (*pfmdr1*) encodes the p-glycoprotein homologue 1, a transporter protein thought to modulate chloroquine accumulation in the digestive vacuole (Valderramos and Fidock, 2006) (Figure 1.7). Polymorphisms in the *pfmdr1* gene have been shown to alter the specificity of the transporter (Sanchez *et al*., 2008). Polymorphisms at codons 86, 184, 1042 and 1246 are associated with reduced sensitivity or resistance to a number of different classes of antimalarials (Dokomajilar *et al*., 2006). The wildtype *pfmdr1* 86 allele is associated with low susceptibility to lumefantrine, halofantrine and mefloquine while the mutant 1246 allele is associated with reduced sensitivity to mefloquine and halofantrine (Dokomajilar *et al*., 2006). Polymorphisms in the *pfmdr1* gene are also associated with chloroquine resistance but in a modulating role rather than conferring chloroquine resistance *per se* (Laufer and Plowe, 2004; White, 2004). Research suggests increased *pfmdr1* copy numbers may be associated with artemether-lumefantrine treatment failure (Price *et al*., 2004; Sisowath *et al*., 2005). Single nucleotide polymorphisms (SNPs) and copy number variations in this gene can therefore be used as markers of lumefantrine tolerance and resistance (Sisowath *et al*., 2009).

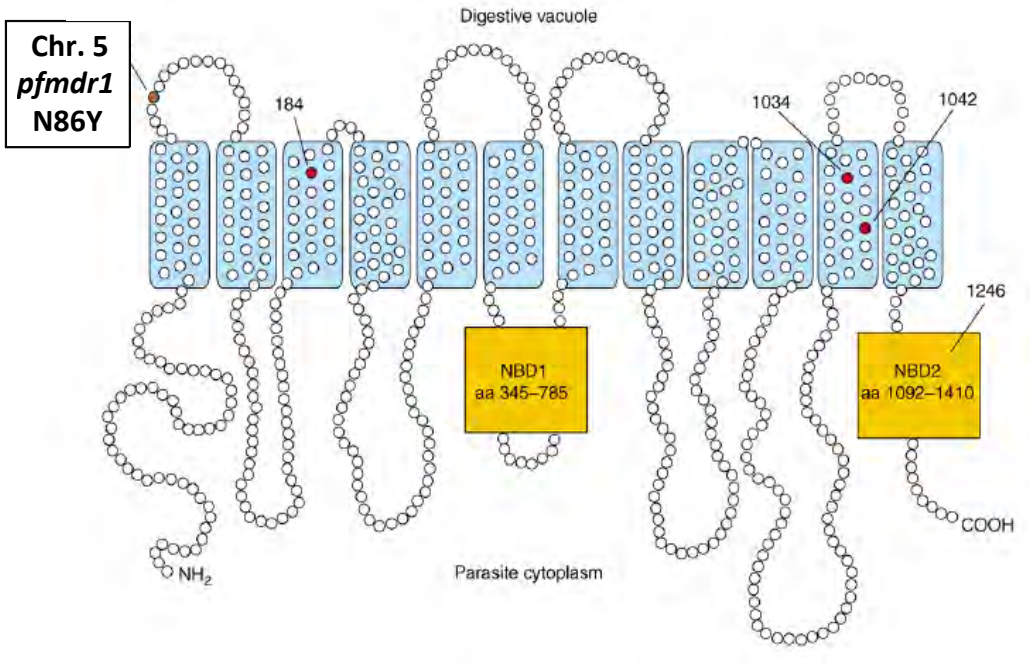


Figure 1.7: Structure of the p-glycoprotein homologue 1 transmembrane protein encoded by the *P. falciparum* multi-drug resistance gene 1 showing the mutations associated with resistance (red filled circles) (adapted from Valderramos and Fidock, 2006).

1.5.3. Evolution and spread of antimalarial drug resistance

There is strong selective pressure on parasites to develop resistance when antimalarial use is widespread and indiscriminate (White and Pongtavornpinyo, 2003). Exposure of parasites to sub-therapeutic concentrations of antimalarial drugs promotes the selection of resistant parasites in the infecting population (White, 1998). Selection can occur from newly acquired infections especially when resistance levels are low (White, 1998). However as resistance levels increase, recrudescence occurs and initial infections are able to withstand the chemical insult of the antimalarial drug (White, 1998). These parasites are more likely to be transmitted because gametocyte carriage is higher in recrudescent and resistant infections increasing the transmission potential of the infection (Barnes and White, 2005; White, 1998).

Various factors contribute to the development and persistence of drug resistance in a parasite population (Esteva *et al*., 2009). Resistance selection pressure may arise from variations in drug dosing rate, infection status of the patient, treatment coverage, clonality, intrahost interactions, drug half-life and immunity (Alexander *et al*., 2007; Anderson and Roper, 2005). In low transmission settings immunity does not develop, consequently individuals suffer symptomatic infections and may seek antimalarial treatment (White, 2004). The need for treatment is therefore higher in low transmission areas resulting in increased drug use and drug pressure in the area; this is

characteristic of Southeast Asia and may explain why this region is a hotspot for the development of antimalarial drug resistance (Maiga *et al*., 2007; Roper *et al*., 2004).

In high transmission areas, malaria specific partial immunity is developed over time (as discussed in section 1.3.4). Infections in individuals with partial immunity are asymptomatic and often go untreated (White, 2004). This lack of treatment seeking behaviour suggests that resistance spread is slower in high transmission settings because of a lack of drug pressure, however mathematical models show that transmission of resistant parasites are not only a factor of transmission intensity but also rate of transmission (Chiyaka *et al*., 2009). High malaria transmission rates are thought to spread resistance and drug resistant parasites are found to be more infective than sensitive ones (Barnes and White, 2005).

1.5.3.1. Chloroquine resistance

Chloroquine was introduced into the eastern parts of Africa in the late 1940s and by 1950 its use was widespread in sub-Saharan Africa (Nuhawa, 2001; Trape, 2001). Chloroquine resistance, measured by clinical reports, was first observed in the late 1970s in East Africa and by 1989 it was firmly established across sub-Saharan Africa (Naidoo and Roper, 2010; Nuwaha, 2001). Chloroquine resistance appears to have emerged independently on at least three separate occasions; in South America, the Pacific and Southeast Asia and subsequently radiated to other parts of the world (Mita *et al*., 2009; Plowe, 2009). The Southeast Asian form of the *pfcrt* mutation spread to East Africa in 1978, twenty years after it arose in Southeast Asia and subsequently spread to the western regions of the African continent by the 1990s (Anderson and Roper, 2005; Mita *et al*., 2009). As a result of widespread chloroquine resistance, Malawi, in 1993, was the first African country to discontinue chloroquine as first-line treatment of uncomplicated malaria and switched to sulphadoxine-pyrimethamine (Kublin *et al*., 2003).

1.5.3.2. Sulphadoxine-pyrimethamine resistance

Pyrimethamine resistance emerged at least 10 years before sulphadoxine resistance (Mita *et al*., 2009; Roper *et al*., 2003). Varied *dhfr* point mutations have been observed and these code for a range of tolerance to pyrimethamine from intermediate to high, depending upon the number of mutations present (Naidoo and Roper, 2011). The *dhfr* S108N mutation is said to have arisen independently on different occasions whilst the *dhfr* triple (108N+51I+59R) mutation appears to have arisen only twice, in South America and Southeast Asia (Anderson and Roper, 2005; Mita *et al*., 2009; Plowe, 2009). Studies have shown that the *dhfr* triple mutation in Africa and Asia share a single common origin, indicative of a selective sweep across the continents (Anderson and Roper, 2005; Gatton and Cheng, 2006; Roper *et al*., 2004) suggesting that gene flow rather than spontaneous mutations drives pyrimethamine resistance (Laufer and Plowe, 2004). Research

suggests that the *dhfr* triple mutation thus migrated from Southeast Asia to Africa (Anderson and Roper, 2005). In most of Africa, the *dhfr* triple mutation (108N+51I+59R) is a highly prevalent resistant allelic combination (Enosse *et al*., 2008; Mita *et al*., 2009). The *dhfr* 164L mutation carries some fitness cost to the parasite which is probably why it is not common (Hyde, 2008) and has been reported mainly in East Africa including Central African Republic, Comoros, Kenya, Madagascar, Malawi, Rwanda, Uganda and is absent in 21 countries (Naidoo and Roper, 2011).

The origin of mutations in the *dhps* gene is still unclear with reports suggesting separate origins in Africa and Southeast Asia (Anderson and Roper, 2005; McCollum *et al*., 2008). The *dhps* double mutation (437G and 540E) is prevalent in southern and East Africa and when combined with the *dhfr* triple mutation, forms a highly resistant quintuple mutation allelic combination that has been associated with SP treatment failure (Pearce *et al*., 2009; Roper *et al*., 2003). The *dhps* 437G and 540E double mutation is the most commonly reported of all *dhps* mutations in southeast Africa and is thought to have a single origin in Africa (Pearce *et al*., 2009).

Sulphadoxine-pyrimethamine as a combined antifolate monotherapy was first introduced in Thailand in the 1960s and subsequently to the rest of Southeast Asia, South America and lastly Africa (Mita *et al.*, 2009; Plowe, 2009). Resistance to sulphadoxine-pyrimethamine emerged in the 1980s in Thailand and quickly spread to South America (Mita *et al.*, 2009). Resistance in Africa has been gradual but by the year 2000 was firmly established in most parts of the continent (Mita *et al.*, 2009; Plowe, 2009).

1.5.3.3. Artemether-lumefantrine resistance

The molecular basis of artemether-lumefantrine resistance has yet to be determined. It was hypothesized that resistance to artemisinins would take a long time to develop because the drug has a short half- life and therefore parasites may not be exposed to sub-therapeutic drug concentrations for extended periods of time. Also, due to its rapid gametocytocidal activity, the potential for transmission is reduced therefore parasites treated with the drug may not successfully infect mosquitoes and spread the disease. Artemisinin derivatives are used in combination with other antimalarials such as lumefantrine and mefloquine to delay the development of resistance (Meshnick, 2002; O'Brien *et al*., 2011). Howevever reports from Southeast Asia of a reduced susceptibility to artemisinin derivatives characterized by slow parasite clearance have emerged (Dondorp *et al*., 2009; Dondorp *et al.*, 2011). This phenomenon is thought to be due to reduced expression of metabolic or cellular pathways in the ring and trophozoite stages with increased expression in the schizont stages (Mok *et al*., 2011). This increased protein synthesis in the schizont stage may counteract the damaging effects of oxidative stress or protein alkylation thought to be associated with artemisinins mode of action (Mok *et al*., 2011). Another proposal to explain

slow parasite clearance times is that a dormancy trait allows parasites to arrest their intra-erythrocytic ring-stage development after artemisinin treatment for weeks before resuming normal cell growth (O'Brien *et al*., 2011). Even though true artemisinin resistance has yet to be defined, selection of tolerance to the treatment may be associated with increased selective pressure on artemisinins (Dondorp *et al*., 2011). This could be considered as a step toward artemisinin resistance. Parasite clearance time is considered a marker for artemisinin resistance and is thought to have a genetic basis (Phyo *et al*., 2012). Plausible molecular markers of artemisinin resistance include transcriptional patterns of genes involved in cell cycle regulation; chromatin remodelling and intracellular signalling (Mok *et al*., 2011).

The selective pressure for resistance to artemether-lumefantrine is thought to be attributed by lumefantrine since it has a longer half-life compared to artemether (Hastings and Ward, 2005). The *pfmdr1* 86N allele has been identified as a marker of lumefantrine tolerance with reports from Uganda (Dokomajilar *et al*., 2006) and Tanzania (Humphreys *et al*., 2007) confirming its selection after artemether-lumefantrine use in Africa (Hastings and Ward, 2005).

1.6. Methods of studying molecular markers of drug resistance in *Plasmodium falciparum*

The following is taken from the World Health Organization guidelines (World Health Organization, 2010b) for approved tools for the measurement of drug resistance:

"The measurement of drug resistance in malaria is complex as four different tools are used:a) therapeutic drug efficacy studies for the measurement of clinical and parasitological efficacy; this method is primarily used by national malaria control programmes in implementing decisions about treatment policy; b) in vitro studies using isolates for the measurement of intrinsic sensitivity to antimalarial drugs; c) pharmacokinetic studies for the characterization of drug absorption and activity in the human body; and d) studies of molecular markers which identify mutations in the parasite genome".

Drug resistance in the present study was measured using molecular markers in the genes encoding drug resistance to chloroquine, sulphadoxine-pyrimethamine and lumefantrine.

1.6.1. *Plasmodium falciparum* DNA

The genome of the *P. falciparum* parasite was sequenced in 2002 (Gardner *et al*., 2002) and consists of 23.3 megabase pairs (Mb) with 14 chromosomes which range from approx. 0.6 to 3.4 Mb in size (Conway, 2007; Sharma, 2005). Extra-chromosomal DNA exists in separate

mitochondrial (approx. 6 kb) and plastid (35 kb) genomes (Conway, 2007; Sharma, 2005). The differentiation of *Plasmodium* spp. can be achieved by amplification of the small-subunit 18S rRNA and circumsporozoite genes (Moody, 2002). The large-subunit RNA gene is highly conserved among *Plasmodium* spp. enabling a genus-specific approach to DNA amplification (Berry *et al*., 2005; Cunha *et al.*, 2009; Moody, 2002). The amplification of mitochondrial DNA by conventional polymerase chain reaction (PCR) has resulted in 100% specificity and sensitivity in one study suggesting its use over nuclear DNA which requires two rounds of amplification and is more costly (Cunha *et al*., 2009).

1.6.2. Genotyping of *P. falciparum* populations

The extraction of parasite DNA is a crucial step in the genotyping process and methods that maximize the amount of DNA yielded are constantly being developed (Bereczky *et al*., 2005). The criteria used when selecting methods for DNA preparations in epidemiological studies are: 1) quick and ease of preparation; 2) high throughput; 3) high reliability; 4) yield of good quality DNA that can be stored for long periods; 5) reduced risk of cross-contamination; and 6) cost efficiency (Henning *et al*., 1999).

Whilst microscopy is a primary method of parasite detection and species identification, its use can be challenging (Conway, 2007). Microscopy can be technically challenging, labour-intensive and inaccurate (Hsiang *et al*., 2010). Various techniques have been used in detecting and genotyping malaria parasites such as erythrocyte lysis, proteinase K digestion and phenol-chloroform extraction (Kain and Lanar, 1991). These methods, however, require either fresh whole-blood samples or freshly frozen and transported venipuncture blood samples. These requirements cannot always be met in field studies (Kain and Lanar, 1991). In addition, these methods have proven to be tedious (Kain and Lanar, 1991). The use of filter paper to store DNA is hailed as being efficient, inexpensive, simple and easy to store and transport (Henning *et al*., 1999; Kain and Lanar, 1991). Since the presence of ions in the blood may inhibit the amplification of plasmodial DNA by PCR (Kain and Lanar, 1991), Chelex-100 is used for DNA extraction since it is an ion exchange resin containing ions that act as chelating groups that bind metal ions (Sepp *et al*., 1994). Removing the metal ions inactivates nucleases and enzymes that destroy DNA, promote DNA degradation and inhibit the function of *Taq* DNA polymerase (Kain and Lanar, 1991; Sepp *et al*., 1994). The Chelex-100 method was found to perform better than other DNA extraction methods when compared in some studies (Hsiang *et al*., 2010; Viana *et al*., 2010). In addition the Chelex-100 method is inexpensive, easily implemented and provides sufficient yield of stable DNA for molecular analyses (Hsiang *et al*., 2010; Polski *et al*., 1998; Viana *et al*., 2010) and was chosen for use in this study.

The polymerase chain reaction has become a useful technique in epidemiological studies where the genotyping of pathogens has become an important source of information, considering the small amounts of genetic material usually available (Henning *et al*., 1999). The polymerase chain reaction is also sensitive in detecting low parasitaemia and mixed species infections (Hsiang *et al*., 2010). Nested-PCR is an alternative PCR amplification technique which requires two successive amplification steps using two different primer pairs (Berry *et al*., 2005). Nested-PCR is often applied to *Plasmodium* detection techniques because of its improved sensitivity and specificity over conventional PCR (Cunha *et al*., 2009). Despite it being more costly and time consuming, the use of nested PCR in malaria epidemiological work is beneficial. It improves the ability to detect minor variants in mixed infections as the limits of detection of parasitaemia are quite low with microscopy (Snounou *et al*., 1999).

Real-time PCR or quantitative-PCR (q-PCR) is another PCR amplification technique used to genotype malaria infections. Quantitative-PCR is a highly sensitive technique which enables the amplification and quantification of specific nucleic acid sequences with detection of the final PCR product in real time (Popa *et al*., 2009). The major advantage that q-PCR holds over the previously described methods is the ability to directly detect specific targets thereby eliminating the need for post-amplification handling of the PCR products such as in agarose gel electrophoresis, radioactive probing or use of ultraviolet light (Dorak, 2006). A major drawback of this technique is its high cost, making it unaffordable in malaria endemic countries.

1.6.3. Multiplicity of infection

In areas of high malaria endemicity, the presence of multiple infections in individual patients has been observed (Henning *et al*., 1999). Markers that are commonly used for *P. falciparum* genotyping are gene regions encoding the three surface antigens *viz.* merozoite surface proteins 1 (msp-1) and 2 (msp-2) and glutamate-rich protein (GLURP) (Farnert *et al*., 2001). The genetic diversity of *P. falciparum* infections can be determined by amplification of the msp-2 gene, a single-copy, highly polymorphic gene used to assess the mean number of genotypes per infection also known as the multiplicity of infection (Bereczky *et al*., 2005; Farnert *et al*., 2001). The msp-2 repeat region is known as block 3 and alleles are classified into two sequence groups; FC27 and ICI 3D7 (Farnert *et al*., 2001).These genes vary in length due to tandemly repeated sequences and alleles can be differentiated by the electrophoresis of PCR products (Farnert *et al*., 2001). The msp-2 marker has been found to be more informative in the detection of multiple infections advocating its use in the present study (Cattamanchi *et al*., 2003; Farnert *et al*., 2001; Snounou *et al*., 1999). The identification of multiple infections is useful in malaria epidemiological studies as it depicts diversity of the parasite population which has implications for antimalarial efficacy,

pathology, acquisition of immunity and transmission potential (Farnert *et al*., 2001; Snounou *et al*., 1999).

1.7. The present study

1.7.1. The Lubombo Spatial Development Initiative (LSDI)

The Lubombo Spatial Development Initiative (LSDI), which was a collaborative agreement between the governments of South Africa, Swaziland and Mozambique, was established in 1999 with the aim of improving the economic and social conditions within this region (Lubombo Spatial Development Initiative, 2010) (Figure 1.8). The Lubombo region (encompassing northern KwaZulu-Natal, eastern Swaziland and southern Mozambique) is characterised by unemployment, poverty and low social economic development (Lubombo Spatial Development Initiative, 2010). The high burden of malaria within the Lubombo region threatened to undermine the success of the initiative (Sharp *et al*., 2007). To address this issue, an integrated malaria control programme was implemented in 1999 (Lubombo Spatial Development Initiative, 2010). Malaria control efforts focused on southern Mozambique as both South Africa and Swaziland had well established and effective national malaria control programmes (Lubombo Spatial Development Initiative, 2010). The control interventions were introduced into Maputo Province in 1999 and based on the successes achieved, were extended into Gaza Province in 2006 (Lubombo Spatial Development Initiative, 2010) (Figure 1.9). The malaria intervention, which included indoor residual spraying with insecticides and artemisinin-based drug treatment, resulted in a decline in malaria transmission rates and malaria case numbers in Maputo Province (from 60% to 10%) and Gaza Province (from 40% to 12%) to date (Lubombo Spatial Development Initiative, 2010).

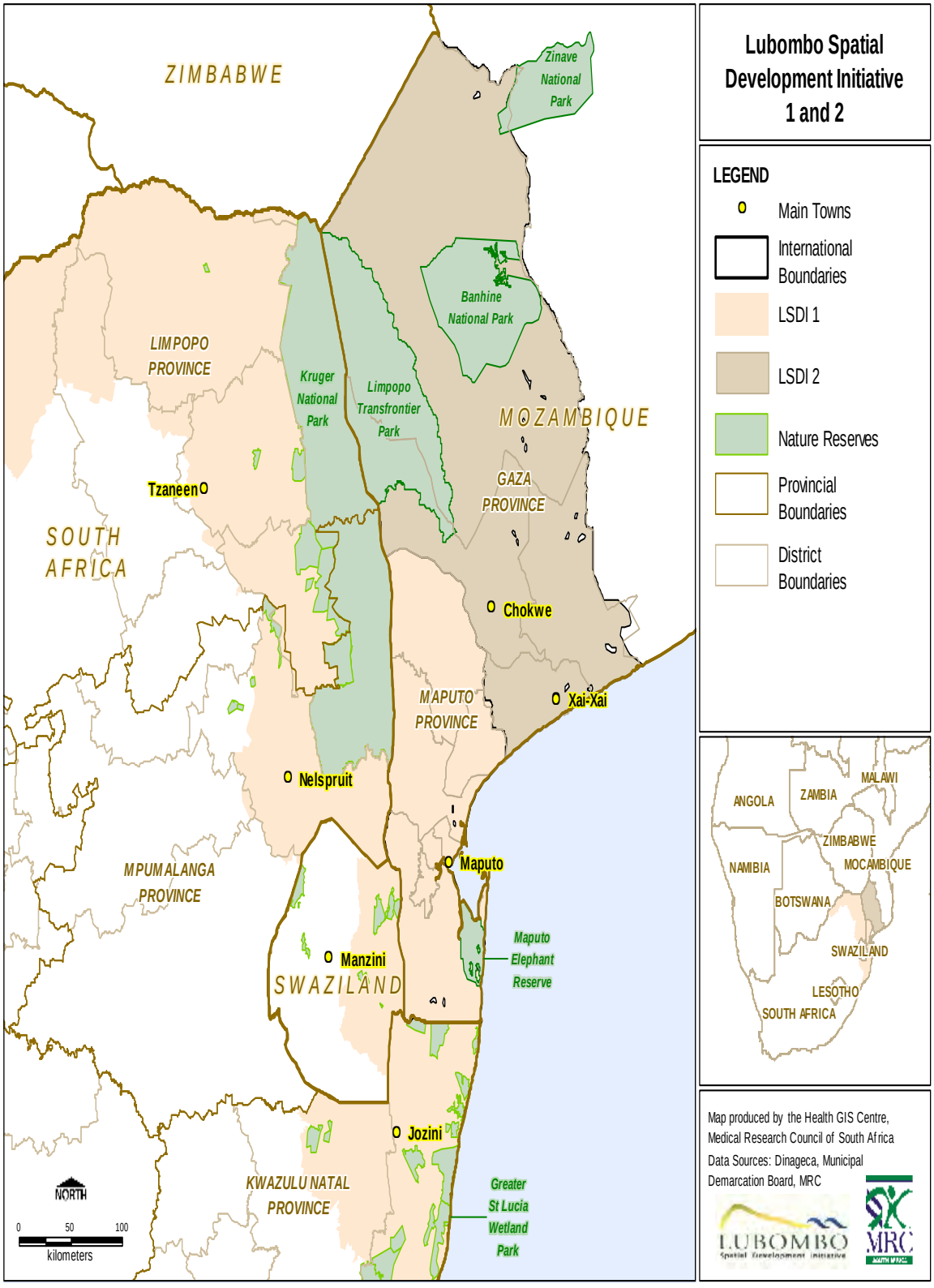


Figure 1.8: The Lubombo Spatial Development Initiatives malaria study sites in South Africa, Swaziland and southern Mozambique.

Figure 1.9: Malaria control zones and study sites in Gaza Province, southern Mozambique.

1.7.2. Study area

This study was conducted in Gaza Province, Mozambique. Mozambique, a southeast African country, borders Tanzania and Malawi to the north, Zambia and Zimbabwe to the west, and South Africa and Swaziland to the south with the Indian Ocean along its eastern border. Malaria transmission is seasonal and generally occurs between November and July (World Health Organization, 2009a). Most malaria cases are caused by *P. falciparum* and the main malaria vectors are *An. gambiae, An. arabiensis* and *An. funestus* (World Health Organization, 2011a). The majority of the reported cases are not diagnostically confirmed (World Health Organization, 2009a).

Despite continued efforts to control the disease, malaria remains a health burden in Mozambique with 9.8 million cases reported in 2006 alone (World Health Organization, 2009a). The principal method of control has been indoor residual spraying, protecting 2 million households and 6.5 million people at risk in 2008 (36% of population at risk) (World Health Organization, 2009a). Long-lasting insecticide treated bed nets were distributed by the national malaria control programme in Mozambique from 2006 to 2008 offering protection to 44% of the population at risk (World Health Organization, 2009a). Another arm of the control initiative included case management with definitive diagnosis using HRP-2 detecting RDTs and treatment with ACTs. More than 6.1 million and 4.8 million ACT treatment courses were delivered by the programme in 2007 and 2008 respectively and these were sufficient to treat all reported cases in the public sector (World Health Organization, 2009a).

1.7.3. Antimalarial drug use in Mozambique

Until 2002, the preferred antimalarial drugs for the treatment of uncomplicated malaria in Mozambique were chloroquine as first-line treatment and sulphadoxine-pyrimethamine as second-line treatment (Allen *et al*., 2009; Raman *et al*., 2008). Following the WHO recommendation that malaria endemic countries move to combination therapy for the treatment of uncomplicated malaria, amodiaquine plus sulphadoxine-pyrimethamine replaced chloroquine as first-line treatment in 2002 (Allen *et al*., 2009; Enosse *et al*., 2008; Fernandes *et al*., 2007). This drug policy was however changed in 2004 when the ACT artesunate plus sulphadoxine-pyrimethamine became the drug of choice in the country (Raman *et al*., 2008). District-level roll out of this drug began in Maputo Province in 2004 (Raman *et al*., 2010) and in Gaza Province in 2008 (Lubombo Spatial Development Initiative, 2009).

Resistance markers associated with sulphadoxine-pyrimethamine resistance have risen markedly in Gaza Province following the introduction of artesunate plus sulphadoxine-pyrimethamine and the use of sulphadoxine-pyrimethamine as a prophylactic in intermittent preventive treatment of pregnant women since 2007 (Alifrangis *et al*., 2003; Allen *et al*., 2009; Fernandes 2007; Lubombo Spatial Development Initiative, 2009; Raman *et al*., 2010). It has been reported that the efficacy of ACTs is severely compromised if their partner drugs are not effective in their own right (Yeka *et al*., 2005). The Mozambican Ministry of Health therefore changed its drug policy in 2008 from artesunate plus sulphadoxine-pyrimethamine to artemether plus lumefantrine (Lubombo Spatial Development Initiative, 2009). Again the drug was first rolled out in Maputo Province, with the first batches of the drug reaching Gaza Province in late 2009.

This dissertation focuses on the effect that changes in antimalarial drug treatment, following changes in treatment policy, have had on the prevalence of molecular markers associated with antimalarial drug resistance in Gaza Province, Mozambique.

1.8. Aims and objectives

The aim of this study is:

To investigate the prevalence of resistant markers associated with sulphadoxine-pyrimethamine, chloroquine and artemether-lumefantrine resistance in six districts across Gaza Province, Mozambique in 2010 and 2011, following the introduction of artemether-lumefantrine as first-line treatment for uncomplicated *falciparum* malaria in 2010.

The research objectives of this study are to:

1. Determine the prevalence of the sulphadoxine-pyrimethamine resistance markers in sentinel sites within Gaza Province, Mozambique during 2010 and 2011 following the withdrawal of artesunate plus sulphadoxine-pyrimethamine.
2. Determine the prevalence of the chloroquine resistance markers in sentinel sites within Gaza Province, Mozambique during 2010 and 2011 following the introduction of ACTs as first-line treatment.
3. Determine the prevalence of resistant markers associated with lumefantrine resistance in sentinel sites within Gaza Province, Mozambique during 2010 and 2011 following the introduction of artemether-lumefantrine as first-line treatment.

CHAPTER 2: Materials and Methods

2.2. Introduction

In this chapter, the study sites, methods of collection and laboratory procedures followed during the course of this project are described. All analyses were performed at the molecular laboratories of the Malaria Research Unit, Medical Research Council unless otherwise stated. Quality control checks of the data were periodically performed by Dr Raman.

2.2. Study area, participants and ethics

2.2.1. Study Area

The present study formed part of the malaria control arm of the Lubombo Spatial Development Initiative in Gaza Province, Mozambique conducted between 2006 and 2011. Cross-sectional community-based parasite prevalence surveys were conducted over a 10 day period during September 2010 and over a 15 day period during July 2011 across Gaza Province, Mozambique.

Gaza Province spans 75 334 km^2 and has a population of *circa.* 1.5 million people (Instituto Nacional de Estatistica, 2008). The main economic activity in the province is agriculture with cashew nut farming an important source of income (Tvedten *et al*., 2010). The province has 11 districts with a population density of 16 per km^2 (Tvedten *et al*., 2010). The present study was conducted at 20 sites in six of the province's districts *viz.* Chokwe, Guija, Massingir, Bilene Macia, Xai-Xai and Xai-Xai Sede (Figure 2.1). These sites were grouped into two study zones, *viz.* Zone 4 and Zone 5 by the LSDI (Table 2.1). The 20 sites within the two zones, termed sentinel sites, were selected based on population size and proximity to health facilities and a national road.

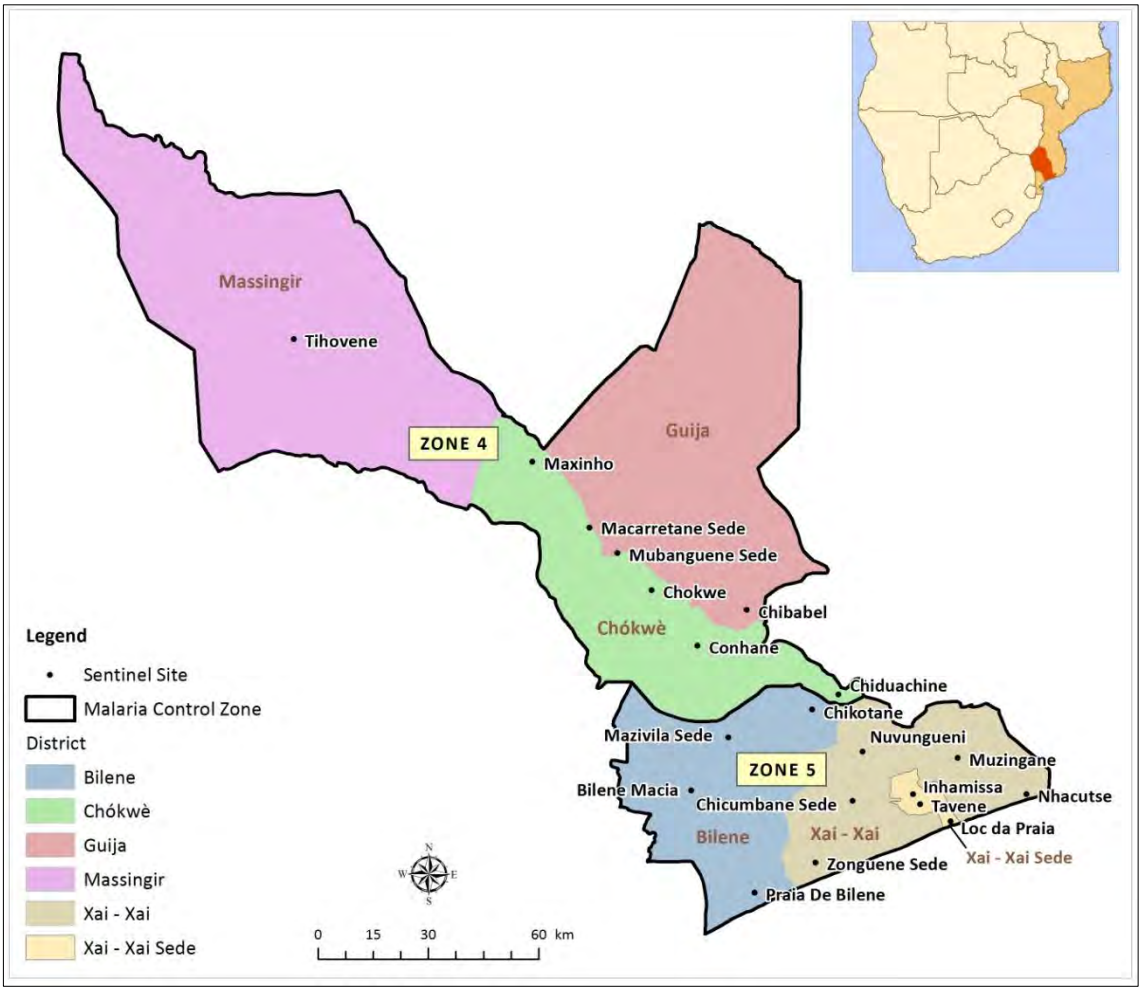


Figure 2.1: Study sites in Gaza Province within six provincial districts and two predefined Lubombo Spatial Development Initiative zones where sample collection occurred.

Table 2.1: Districts and study sentinel sites classified into predefined Lubombo Spatial Development Initiative zones in Gaza Province

LSDI zone	Districts	Number of sentinel sites
Zone 4	Chokwe; Guija; Massingir	8
Zone 5	Xai Xai; Xai Xai Sede; Bilene	12

2.2.2. Participants

At each site, 150 asymptomatic children between the ages of two and less than fifteen years were randomly recruited for participation in the survey and were tested for malaria, using HRP-2 detecting RDTs, once consent was obtained from a parent/guardian. Children who had a positive

RDT result for malaria were either given artemether-lumefantrine or referred to the nearest health facility.

2.2.3. Ethics

Ethical approval for this study was obtained from the South African Medical Research Council and the Provincial Directorate of Health in Gaza Province, Mozambique as part of the Lubombo Spatial Development Initiative (Global Fund Grant MAF-22-GO2-M-00).

2.3. Sample collection

Each study participant was tested for a *P. falciparum* malaria infection using a rapid diagnostic test (RDT) designed to detect *P. falciparum* HRP-2 (SD Bioline; Malaria Ag P.f (*Pf*HRP-2), Standard Diagnostics, Inc., Korea). For each participant, gender and age were recorded, auxiliary temperature was taken using a digital thermometer, the RDT result was recorded and finger-prick blood was blotted onto a filter paper strip (3MM Whatman filter paper, Merck Laboratory Supplies (Pty) Ltd., Durban, South Africa). Each filter paper blood spot sample was air-dried and individually packed in air-tight zip-lock packets containing desiccant. Upon completion of the survey, samples were transported to the molecular biology laboratories of the Malaria Research Unit in Durban, South Africa and stored at room temperature until molecular analyses were conducted.

2.4. DNA extraction

2.4.1. Reagents

1 M HCl: 86.5 ml of 37% HCl was dissolved in 1000 ml of distilled water (dH_2O).
10 X Phosphate buffered saline (PBS) buffer: 80 g of NaCl, 2 g of KCl, 14.4 g of Na_2HPO_4 and 2.4 g of KH_2PO_4 were dissolved in 1000 ml dH_2O. The pH was adjusted to 7.4 with 1 M HCl.
PBS buffer/5% SDS solution: 10 ml of PBS buffer and 5 ml of SDS were dissolved in 85 ml dH_2O.
1 X PBS washing solution: 10 ml of PBS was dissolved in 90 ml dH_2O.
20% Sodium dodecyl sulphate: 40 g of electrophoresis-grade SDS was dissolved in 200 ml dH_2O. The solution was heated to 68 ^{0}C and gently stirred.
5 M NaOH: 200 g of NaOH was dissolved in 1000 ml dH_2O.
20 % Chelex solution: 40 g of Chelex-100 was dissolved in 200 ml dH_2O. The pH was adjusted to 9.5 using 5 M NaOH.

2.4.2. Procedure

Parasite DNA was extracted from RDT positive filter paper blood spot samples using the Chelex-100 method of Wooden *et al*. (1993). Briefly, approximately ¼ of a blood spot was cut using flame sterilized scissors and placed into labelled 1.5 ml Eppendorf tubes containing 1 ml of PBS buffer/5% SDS solution. The tubes, containing the buffer and bloodspot, were briefly vortexed and incubated overnight at 37 ºC in a waterbath to allow cell lysis to occur. The following morning, the samples were removed from the waterbath and inverted twice after which the PBS buffer was discarded ensuring the filter paper remained in the tube. The tubes containing the paper were centrifuged (1 min at 14 000 rpm; Eppendorf model 5424) and remaining buffer was removed using a pipette. One ml of 1 X PBS washing buffer was added to each tube to remove red blood cell residue. After inverting twice, the washing buffer was discarded ensuring the filter paper remained in the tube. The tubes were then centrifuged (1 min at 14 000 rpm; Eppendorf model 5424) and remaining buffer was removed using a pipette. To each filter paper containing tube, 150 µl of autoclaved distilled water (dH_2O) and 50 µl of Chelex (20% m/v) was added and the tube was boiled over steam for 10 minutes. After boiling, the tubes were cooled on ice then centrifuged (1 min at 14 000 rpm; Eppendorf model 5424) to allow for Chelex and supernatant separation. The supernatant containing the *Plasmodium* DNA was carefully removed with a pipette and placed in a new, clean, autoclaved, labelled 0.5 ml Eppendorf tube. Isolated DNA was stored at – 20 ^{0}C until required.

2.5. DNA genotyping

2.5.1. General reagents and equipment

All samples were analysed in a 96-well format using PCR plates (Starlab Group, International; Axygen, California, USA) and sealed with heat sealing foil (Eppendorf) or strip caps (Axygen, California, USA).

2.5.2. *Plasmodium* species identification

The extracted parasite DNA was subjected to two PCR methods to determine the parasite species present in each sample. The two methods used were the quantitative-PCR (q-PCR) protocol described by Mangold *et al*., (2005) and the nested PCR protocol of Snounou *et al*., (1993).

2.5.2.1. The q-PCR method of identifying *Plasmodium* species by Mangold *et al.* (2005)

In this q-PCR method, the 18S rRNA of the parasite was targeted as it contains highly conserved and variable regions. Each 25 µl q-PCR reaction contained 5 µl of sample DNA, 12.5 µl of BioRad IQ SYBRGreen Supermix, 1 µl each of forward and reverse primer (20 µM) (Table 2.2) and 5.5 µl of dH_2O. Primers were synthesized by Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa. Samples were centrifuged (5 mins at 3 000 rpm; Thermo Electron Corporation, Jouan B4i multifunction centrifuge) before q-PCR and analyzed in a BioRad IQ5 multicolor real-time PCR detection system. The thermal cycling conditions consisted of initial denaturation at 95 ºC for 10 minutes; followed by 50 cycles of denaturation at 95 ºC for 10 seconds, annealing at 50 ºC for 30 seconds and extension at 72 ºC for 70 seconds. Amplification was immediately followed by a melt program of 0.5 ºC increments from 55-95 ºC with a 30 second dwell time. Melt curve analysis and temperature dissociation curves enabled the differentiation between four *Plasmodium* species. The melting temperatures of each *Plasmodium* species are presented in Table 2.3. The plates included a negative control to ensure that amplification was not due to contaminating DNA. Negative controls contained all reaction reagents with the exception of DNA. All amplifications were run in duplicate, with a third run conducted on all discordant samples.

Table 2.2: Consensus primers used in the amplification and detection of *P. falciparum* DNA

Primer	Primer sequence (5' to 3')
PL1473F18 (forward)	TAACGAACGAGATCTTAA
PL1679R18 (reverse)	GTTCCTCTAAGAAGCTTT

Table 2.3: Range of melting temperatures of dissociation curves used in the Mangold *et al.* (2005) method for distinguishing *Plasmodium* species

***Plasmodium* species**	**Temperature range of melt curve**
Plasmodium malariae	73.5 ºC to 75.5 ºC
Plasmodium falciparum	75.5 ºC to 77.5 ºC
Plasmodium ovale	77.5 ºC to 79.5 ºC
Plasmodium vivax	79.5 ºC to 81.5 ºC

2.5.2.2. The nested-PCR method for distinguishing *Plasmodium* species by Snounou *et al.* (1993)

In this nested PCR method, both primary and secondary amplifications required the same reagents at the same concentrations; 18 µl reaction mixtures contained 5 µl of Promega GoTaq Flexi buffer

(5 x), 1.8 µl of Promega $MgCl_2$ (25mM), 2.2 µl each of forward and reverse primer (20 µM) (Table 2.4), 1 µl dNTPS (5 mM), 0.15 µl Promega GoTaq Flexi DNA Polymerase (5units/µl) and 6 µl of dH_2O. Primers were synthesized by Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa. For the primary PCR, 3 µl of DNA was added to the reaction mixture whilst 4 µl of this primary PCR product was used as a template in the secondary nested PCR. The volume of dH_2O added to the reaction mixture was adjusted to allow for this change in volume. Samples were centrifuged (1 minute at 3 000 rpm; Thermo Electron Corporation, Jouan B4i multifunction centrifuge) prior to amplification. The thermal cycling conditions consisted of an initial denaturation of 95 ºC for 5 minutes, 30 cycles of denaturation at 94 ºC for 1 minute, annealing at 58 ºC for 2 minutes, extension at 72 ºC for 2 minutes and a final extension step at 72 ºC for 5 minutes. The thermal cycling conditions for both nests were the same. The plates included a negative control to ensure that amplification was not due to contaminating DNA. Negative controls contained all reaction reagents with the exception of DNA. All amplifications were run in duplicate, with a third run conducted on all discordant samples. The PCR products were run on agarose gels as described in section 2.5.2 below, alongside a 100 base pairs (bp) DNA ladder (Fermentas). The banding patterns were analysed, scored and entered into a database; *P. falciparum* positive samples produced bands that were 205 bp in length.

Table 2.4: Primers used for identification of *P. falciparum* (Snounou *et al*., 1993)

	Primer	Primer sequence (5' to 3')	Product size (bp)
First nest	rPLU6	TTAAAATTGTTGCAGTTAAAAGC	1200
	rPLU5	CCTGTGTTGCCTTAAACTTC	
Second nest	rFAL1	TTAAACTGGTTTGGGAAAACCAAATATATT	205
	rFAL2	ACACAATGAACTCAATCATGACTACCCGTC	

2.5.3. Agarose gel electrophoresis of PCR products

2.5.3.1. Reagents

50 X TAE buffer: 242 g of Tris Base, 57.1 ml of glacial acetic acid and 18.6 g of EDTA were dissolved in 1000 ml dH_2O.

0.1 mg/ml Ethidium bromide: 500 mg of Ethidium bromide was dissolved in 50 ml dH_2O.

2 % Agarose gels: 4 g of agarose was added to 200 ml of 1 X TAE buffer and heated in a microwave. After cooling, 10 µl of 0.1 mg/ml ethidium bromide was added to the gels and allowed to mix. Agarose solutions were poured into casting trays and allowed to set until firm and pearly white.

1 X TAE running buffer: 20 ml of 50 X TAE buffer was dissolved in 1000ml dH_2O.

2.5.3.2. Procedure

PCR products were run on 2% agarose gels alongside negative, positive and no template controls. The agarose gels were placed in electrophoresis chambers and submerged in 1 X TAE running buffer. Gels were electrophoresed at approximately 180 V until there was sufficient separation of bands. The gels were visualized and digitally photographed using a MiniBis documentation system (BioSystematica, Ceredigion Wales, UK). Images were stored as tiff files.

2.5.4. Detection of multiplicity of infection

To determine the multiplicity of the *P. falciparum* infections, the nested-PCR method targeting the msp-2 marker as described by Ranford-Cartwright *et al*., (1997) was used. In both rounds of amplification, 10 µl reaction mixtures contained 3 µl of Promega GoTaq Flexi buffer (5 x), 0.8 µl of Promega $MgCl_2$ (25mM), 0.2 µl each of forward and reverse primer (20 µM) (Table 2.5), 0.3 µl dNTPS (5 mM), 0.1 µl Promega GoTaq Flexi DNA Polymerase (5unit/µl), 0.8 µl of glycerol and 5 µl of dH_2O. Primers were synthesized by Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa. For the primary PCR, 3 µl of DNA was added to the reaction mixture whilst 4 µl of this primary PCR product was used as a template in the secondary nested PCR. The thermal cycling conditions for the first round of amplification consisted of an initial denaturation of 95 ºC for 5 minutes, 30 cycles of denaturation at 94 ºC for 25 seconds, annealing at 50 ºC for 30 seconds, extension at 65 ºC for 2 minutes and a final extension step at 72 ºC for 2 minutes. The thermal cycling conditions for the second nest were the same as the first nest except that annealing occurred at 50 ºC for 60 seconds. The plates included a negative control to ensure that amplification was not due to contaminating DNA. Negative controls contained all reaction reagents with the exception of DNA.

Table 2.5: Primers used for the multiplicity of infection analysis (Ranford-Cartwright *et al*., 1997)

	Primer	Primer sequence (5' to 3')
First nest	MSP2S3	GAAGGTAATTAAAACATTGTC
	MSP2S2	GAGGGATGTTGCTGCTCCACAG
Second nest	MSP2S1	GAGTATAAGGAGAAGTATG
	MSP2S4	CTAGAACCATGCATATGTCC

All amplifications were run in duplicate, with a third run conducted on all discordant samples. Amplified products were run on agarose gels as described in section 2.5.2, alongside a 100bp DNA ladder (Fermentas). The banding patterns were analysed, scored and the number of infections per sample were entered into a database. Each band represented an individual *P. falciparum* clonal infection. Images were stored as tiff files.

2.5.5. Amplification of the *dhfr, dhps* and *pfmdr1* genes

The procedures described in the following sections were used to genotype *P. falciparum* isolates to determine the prevalence of resistance markers in the *dhfr, dhps* and *pfmdr1* genes. The protocols followed for the amplification of the *dhfr, dhps* and *pfmdr1* genes was that of Plowe and colleagues described at (http://medschool.umaryland.edu/CVD/2002_pcr_asra.asp).

Samples were assessed for polymorphisms in four codons (51, 59, 108 and 164) of the *dhfr* gene, four codons (436, 437, 540 and 581) of the *dhps* gene and one codon (86) of the *pfmdr1* gene using a nested-PCR method. For both rounds of amplification, 20 µl master mixtures contained 5 µl of Promega GoTaq Flexi buffer (5 x), 2.2 µl of Promega $MgCl_2$ (25mM), 2.2 µl each of forward and reverse primer (20 µM) (Table 2.6), 1 µl dNTPS (5 mM), 0.1 µl *Taq* Promega GoTaq Flexi DNA Polymerase (5unit/µl), and 6 µl of dH_2O. For the *pfmdr1* 86 and *dhfr* 51/59 codons, master mixtures contained 0.15 µl of *Taq* polymerase (5unit/µl). Primers were synthesized by Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa. For the primary PCR, 3 µl of DNA was added to the reaction mixture whilst 4 µl of this primary PCR product was used as a template in the secondary nested PCR; the volume of dH_2O added to the reaction mixture was adjusted to allow for this change in volume. The thermal cycling conditions for the first nest consisted of an initial denaturation of 95 ºC for 5 minutes, 45 cycles of denaturation at 92 ºC for 30 seconds, annealing at 45 ºC for 30 seconds, extension at 65 ºC for 45 seconds and a final extension step at 72 ºC for 5 minutes. The thermal cycling conditions for the second nest were the same for the first nest except that annealing occurred at 42 ºC for 30 seconds and 25 cycles.

Table 2.6: Primers used for the amplification of molecular markers in the *dhfr, dhps* and *pfmdr1*genes

	Primer	Primer name	Primer Sequence (5' to 3')
***dhfr* codons 51 and 59**	Primary PCR forward	FR519-A	GCGCGCTAATAACTACACATTTA
	Primary PCR reverse	FR519-B	CCCGGGCTCTTATATTTCAATTT
	Secondary PCR forward	FR51-D	CTAGGAAATAAAGGAGTATTACCATGGAAATGGA
	Secondary PCR reverse	FR59-D	ATTTTTCATATTTTGATTCATTCACATATGTTGTAACTGTAC
***dhfr* codons 108 and 164**	Primary PCR forward	FR100-A	GGGGGGCAGTTACAACATATGTGA
	Primary PCR reverse	FR100-B	GGGGGCACATTCATATGTACTATTT
	Secondary PCR forward	FR108-D	CTAATTCTAAAAAATTACAAAATGT
	Secondary PCR reverse	FR164-D3	TTTCTTTTCTAAAAATTCTTGATAAACAACGGAACCTCTTA
***dhps* codons 436 and 437**	Primary PCR forward	PS400-A	GGGGTATTAAATGTTAATTATGATTCT
	Primary PCR reverse	PS400-B	GGGGTCACATTTAACAATTTTATT
	Secondary PCR forward	PS400-D1	TGTTCAAAGAATGTTTGAAATGA
	Secondary PCR reverse	PS400-D2	CCATTCTTTTTGAAATAATTGTAAT
***dhps* codons 540 and 581**	Primary PCR forward	PS500-A	GGGCCCAAACAAATTCTATAGTG
	Primary PCR reverse	PS500-B	GGCCGGTGGATACTCATCATATA
	Secondary PCR forward	PS500-D1	GCGCGCGTTCTAATGCATAAAAGAGG
	Secondary PCR reverse	PS500-D2	CCCGGGTAAGAGTTTAATAGATTGATCAGCTTTCTTC
***pfmdr1* codon 86**	Primary PCR forward	MDR-A	GCGCGCGTTGAACAAAAAGAGTACCGCTG
	Primary PCR reverse	MDR-B	GGGCCCTCGTACCAATTCCTGAACTCAC
	Secondary PCR forward	MDR-D1	TTACCGTTTAAATGTTTACCTGC
	Secondary PCR reverse	MDR-D2	CCATCTTGATAAAAAACACTTCTT

The plates included a negative control to ensure that amplification was not due to contaminating DNA. Negative controls contained all reaction reagents with the exception of DNA. All amplifications were run in duplicate, with a third run conducted on all discordant samples.

2.5.6. Restriction enzyme digestion of nested-PCR product for mutation identification in the *dhfr*, *dhps* and *pfmdr1* genes

An established restriction digestion method described by Plowe and colleagues at (http://medschool.umaryland.edu/CVD/2002_pcr_asra.asp) was used to determine the presence of mutations in the *dhfr, dhps* and *pfmdr1* genes using restriction enzymes (New England Biolabs) (Table 2.7). Five µl of PCR product was incubated with 4.5 µl of dH_2O, 1 µl of restriction buffer, 0.1 µl of bovine serum albumin (BSA) and 0.1 µl of restriction enzyme. The restriction enzymes used were EcoRI for *dhfr* 51, BsrGI for *dhfr* 59, AluI for *dhfr* 108, PsiI for *dhfr* 164, MspA1I for *dhps* 436, AvaII for *dhps* 437, FokI for *dhps* 540, MwoI for *dhps* 581 and Af1III for *pfmdr1* 86. Restriction digestion products were separated by agarose gel electrophoresis as described in section 2.5.2. The migration of the samples in the gel was compared to that of non-digested PCR product.

Table 2.7: Restriction enzymes, their cleave sites and product sizes before and after cleavage. The codons representing wildtype and mutant alleles located within the *dhfr* and *dhps* genes are also shown. Amino acid abbreviations and codes are shown in brackets.

Gene	***Dhfr***				***Dhps***				***pfmdr1***
Codon	**51**	**59**	**108**	**164**	**436**	**437**	**540**	**581**	**86**
Product sizes before cleavage (bp)	113	113	254	254	148	148	201	201	291
Enzyme	EcoRI	BsrGI	AluI	PsiI	MspA1I	AvaII	FokI	MwoI	AflIII
Cleave site	G↓AATTC	T↓GTACA	AG↓CT	TTA ↓TAA	CMG↓CKG*	G↓ GWCC**	GGATGN$_9$↓***	GCNNNNN↓NNGC***	A↓ CRYGT****
Product sizes after cleavage (bp)	35 and 78	48 and 65	44 and 210	40 and 214	69 and 79	69 and 79	51 and 150	47 and 154	125 and 166

Table 2.7 cont.: Restriction enzymes, their cleave sites and product sizes before and after cleavage. The codons representing wildtype and mutant alleles located within the *dhfr* and *dhps* genes are also shown. Amino acid abbreviations and codes are shown in brackets.

Allele cleaved	Wildtype	Wildtype	Wildtype	Wildtype	Mutant	Mutant	Mutant	Wildtype	Mutant
Wildtype codon	Asparagine (Asn) (N)	Cysteine (Cys) (C)	Serine (Ser) (S)	Isoleucine (lle) (I)	Serine (Ser) (S)	Alanine (Ala) (A)	Lysine (Lys) (K)	Alanine (Ala) (A)	Asparagine (Asn) (N)
Code	AAT AAC	TGT TGC	AGT AGC	ATT ATC ATA	AGT AGC	GCT GCC GCA GCG	AAA AAG	GCT GCC GCA GCG	AAT AAC
Mutant codon	Isoleucine (Ile) (I)	Arginine (Arg) (R)	Asparagine (Asn) (N)	Leucine (Leu) (L)	Alanine (Ala) (A)	Glycine (Gly) (G)	Glutamic Acid (Glu) (E)	Glycine (Gly) (G)	Tyrosine (Tyr) (Y)
Code	ATT ATC ATA	CGT CGC CGA CGG	AAT AAC	TTA TTG	GCT GCC GCA GCG	GGT GGC GGA GGG	GAA GAG	GGT GGC GGA GGG	TAT TAC

*M: A or C; K: G or T **W: A or T ***N: A or C or G or T ****R: A or G; Y: C or T

2.5.7. Amplification of the *pfcrt* gene

The q-PCR method described by Sutherland *et al*. (2007) was used to genotype the *pfcrt* resistance allele. Probes representing the *pfcrt* CVMNK (wildtype) and *pfcrt* CVIET (mutant) haplotypes were used. Each 25 µl q-PCR reaction contained 2 µl of sample DNA, 12.5 µl of BioRad IQ Supermix, 1 µl of forward and reverse primer each (10 µM) (Table 2.8), 0.3 µl of each probe (Table 2.8) and 8 µl of dH_2O. The samples were centrifuged for 5 minutes before q-PCR. Samples were analyzed in a 96-well format in a BioRad IQ5 multicolor real-time PCR detection system. The thermal cycling conditions consisted of initial denaturation at 95 ºC for 10 minutes; followed by 45 cycles of denaturation at 95 ºC for 15 seconds and annealing at 55 ºC for 60 seconds.

Table 2.8: Primers and probes used in the q-PCR amplification and detection of molecular markers in *pfcrt* codons 72-76

		Sequence (5' to 3')
Primers	PFCRT F (forward)	TGGTAAATGTGCTCATGTGTTT
	PFCRT R (reverse)	AGTTTCGGATGTTACAAAACTATAGT
Probes	crt76CVMNK wildtype	FAM-TGTGTAATGAATAAAATTTTTGCTAA-BHQ1
	crt76CVIET resistant	HEX-TGTGTAATTGAAACAATTTTTGCTAA-BHQ1

2.6. *pfmdr1* copy number amplification

This assay was performed by Dr. J. Raman. To estimate *pfmdr1* copy number in *P. falciparum* isolates, the relative quantification method, TaqMan real-time PCR, of Price *et al*. (2004) was used. Briefly, using q-PCR, the relative gene copy number of *pfmdr1* for each sample was compared to that of a reference gene (β-tubulin) using specific fluorescently labelled probes FAM and VIC (Table 2.9). Amplification reactions were done as multiplex PCR in 96 well plates in 25 µL, containing TaqMan buffer (8% glycerol, 0·625 U DNA polymerase, 5·5 mmol/L MgCl2, 300 µmol/L dNTP, 600 nmol/L passive reference dye ROX (5-carboxy-X-rhodamine), pH 8·3), 300 nmol/L of each forward and reverse primer (Table 2.9), 100 nmol/L of each probe, and 5 µL of template DNA. Fifty cycles were performed (95^0C for 15 s and at 58^0C for 1 min).

Table 2.9: Primers and probes used in the TaqMan copy number amplification assay

		Sequence (5' to 3')
Primers	Forward	TGCATCTATAAAACGATCAGACAAA
	Reverse	CGTGTGTTCCATGTGACTGT
Probes	FAM	TTTAATAACCCTGATCGAAATGGAACCTTTG
	VIC	TAGCACATGCCGTTAAATATCTTCCATGTCT

2.7. Scoring of agarose gels

All gels were scored manually. Samples were classified as either pure sensitive, pure mutant or mixed mutant/wildtype depending on the band present. Mixtures were classified when two bands (both wildtype and mutant genotypes) were present in one sample. When one band was darker than the other, the score given included the amino acid of the visually darker band on the gel. If two bands were of the same colour intensity, a mixed call was given without recording the amino acid. Genotyping assays were run in duplicate with a third run conducted on samples that showed discordant results. Mixed infections were grouped with pure mutant infections in calculations of overall prevalence. A database was created in Microsoft Excel to record all the information for statistical analysis.

2.8. Statistical analysis

Statistical analysis was performed using Stata Intercooled version 11.0 (Stata Corporation, College Station, Texas). Descriptive statistics were first generated. Continuous variables (age) were summarized using means and medians where appropriate. The difference in mean age between zones was analyzed using a t-test. Four categorical variables *viz.* study year, gender, febrility and residence were analyzed by presenting relative frequencies and percentages using cross-tabulations and the association between two categorical variables were tested using Pearson's Chi-squared test and Fishers exact test where small cell frequencies were observed. For these comparisons, odds ratios (OR), p-values and 95% confidence limits were recorded. Next, univariate analyses and multiple variable logistic regressions were performed to determine the effect of the age of participants, gender, febrility, residence in a peri-urban area vs. rural area, zone, and study year on the prevalence of each mutation (*dhfr*, *dhps*, SP quintuple, *pfcrt* and *pfmdr*1). The strength of association was evaluated by computing ORs. The OR is a relative measure of risk indicating the likelihood of the outcome occurring in patients that are exposed compared to those that are not. A p-value less than 0.05 was considered statistically significant and confidence limits were set at 95%. There were 20 sentinel sites in the study, and this was accounted for in the analysis by

specifying the survey design with the “svyset” procedure in Stata. Statistical support was provided by the Biostatistics Unit of the Medical Research Council.

CHAPTER 3: Asexual parasite prevalence and sulphadoxine-pyrimethamine resistance marker prevalence in Gaza Province

3.1. Introduction

The ultimate goal of resistance surveillance is to ensure that all malaria patients are treated with efficacious drugs, and that antimalarial drugs currently in use remain effective for as long as possible (Guerin *et al*., 2009*).* The efficacy of a drug is dependent on a number of factors including acquired immunity, pharmacokinetics and genetics, consequently the predictive value of drug efficacy studies such as *in vivo* studies and molecular markers of resistance are not absolute (Guerin *et al.*, 2009; Laufer *et al*., 2007). *In vivo* drug efficacy studies are the gold standard for assessing antimalarial drug efficacy because individual patient response to the drugs can be closely monitored (Ruebush *et al.*, 2003). These studies are, however, complex, time consuming, labour intensive and expensive and may not always be feasible to conduct (Ruebush *et al.*, 2003). Drug resistance monitoring is a useful step in any successful malaria control intervention. The monitoring of molecular markers of antimalarial drug resistance is a relatively cheap and simple alternative for assessing drug efficacy at the population level, making it a suitable and valuable approach to large-scale mapping and monitoring of malaria control interventions (Guerin *et al.*, 2009; Zakeri *et al*., 2010). Numerous case studies have shown that the inclusion of molecular marker monitoring in integrated malaria control interventions have been beneficial in reducing the malaria burden (Kokwaro, 2009; Vidjaykadga *et al.*, 2006).

Great advances have been made in characterizing the molecular basis of *P. falciparum* drug resistance (Nkhoma *et al*., 2007). The sequencing of the *P. falciparum* genome (Gardner *et al*., 2002) has contributed to the identification of genetic differences between drug sensitive and drug resistant parasites. These differences, manifested as point mutations or variations in gene copy number, have become validated molecular markers of antimalarial drug resistance (Laufer *et al.*, 2007). The effectiveness of a malaria intervention can be assessed by determining the parasite prevalence (the proportion of people infected with malaria parasites) in defined sample populations by active surveillance which involves cross-sectional surveys of defined sample populations (Hay *et al*., 2008). Measurement of parasite prevalence also enables the detection of asymptomatic malaria infections that may serve as a reservoir of transmission (Alves *et al*., 2005; Hay *et al*., 2008). Parasite prevalence is often measured by microscopy, the gold standard, however rapid diagnostic tests (RDTs) are better suited to field conditions during cross-sectional surveys due to their relative ease of transportation and results are available within a few minutes (Wongsrichanalai *et al*., 2007).

In this chapter, the asexual parasite prevalence and sulphadoxine-pyrimethamine (SP) resistance marker prevalence will be presented and discussed, together with their associations with prospectively defined malaria risk factors including; the age of participants, gender (female vs. male), febrility (temperatures of 37.5 ^{0}C and above vs. those at 37.4 ^{0}C and below) and residence (in a peri-urban area vs. rural area).

3.2. Results

3.2.1. Descriptive statistics

Over the two year study period, 5947 children, 2346 from Zone 4 and 3601 from Zone 5, were assessed for malaria using RDTs (Table 3.1). Of the children surveyed, 13.45% (800/5947) were RDT positive for *P. falciparum* malaria. Unfortunately17 finger prick filter paper blood spots were lost during transportation and handling so only 783 (97.88%) RDT positive samples were analysed further in the laboratory. Of these RDT positive samples, 76.11% (596/783) were confirmed as *P. falciparum* positive by nested PCR.

Table 3.1: Total number of participants surveyed in Zones 4 and 5 in 2010 and 2011

	2010		**2011**	
	Total number of participants	**% RDT positive**	**Total number of participants**	**% RDT positive**
Zone 4	1144	7.26	1780	6.63
Zone 5	1202	19.05	1821	21.20
Total	2346	26.31	3601	27.83

The median age of the PCR-confirmed *P. falciparum* subjects in both zones was seven years over the study period (Figure 3.1). Female participants accounted for 51.9% of the PCR-confirmed *P. falciparum* subjects. Only 1.4% of PCR-confirmed *P. falciparum* samples were febrile (auxiliary temperature $\geq$ 37.5 ^{0}C). Due to its low prevalence in the study population, febrility was excluded as a risk factor in all further analysis.

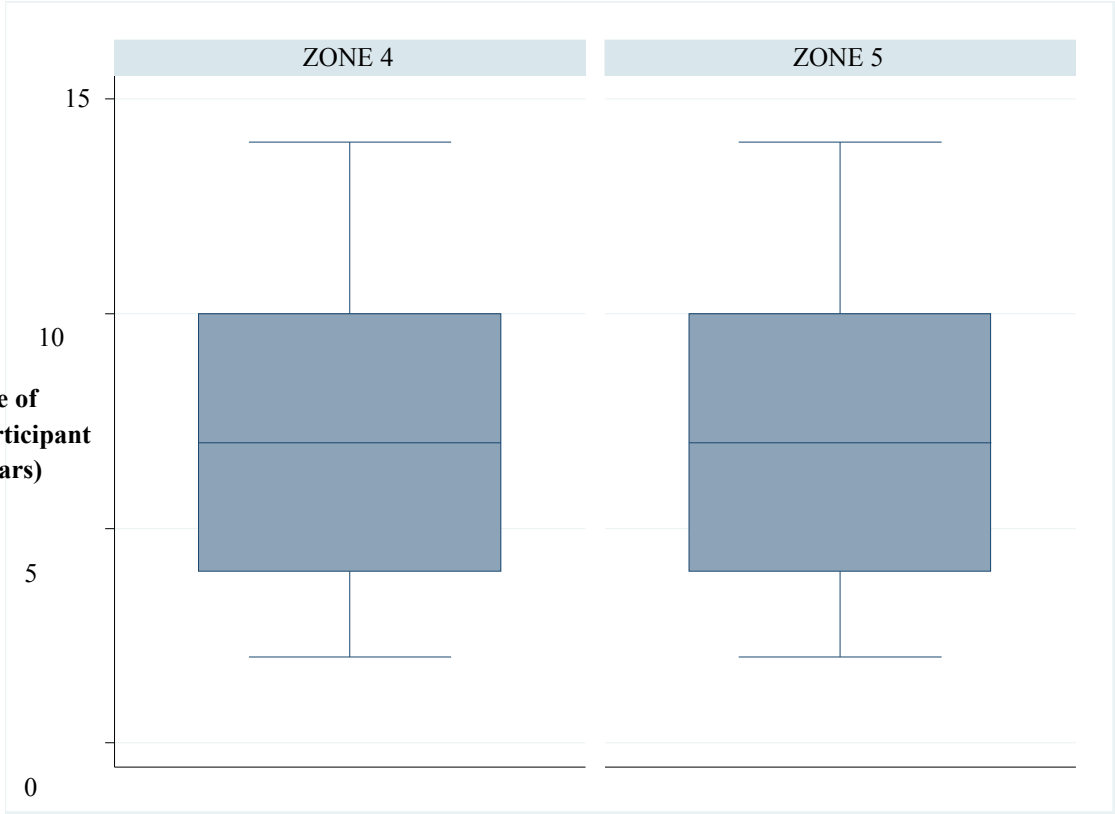


Figure 3.1: Age distribution of PCR-confirmed *P. falciparum* positive study participants surveyed in Zones 4 and 5 over the study period.

A total of 20 sentinel sites were surveyed, eight of which were in Zone 4 and 12 in Zone 5. Of these 20 sentinel sites, 14 were rural and six were peri-urban. There was an equal number of rural sentinel sites between Zone 4 and Zone 5 (Table 3.2). Of the six peri-urban sites, only one of these occurred in Zone 4 (Table 3.2). Rural sites yielded more *P. falciparum* positive samples than peri-urban sites (Figure 3.2).

Table 3.2: Categorization of sentinel sites in Zones 4 and 5 by rural/peri-urban status

	Zone 4	Zone 5	Total
Rural	7	7	14
Peri-urban	1	5	6
Total	8	12	20

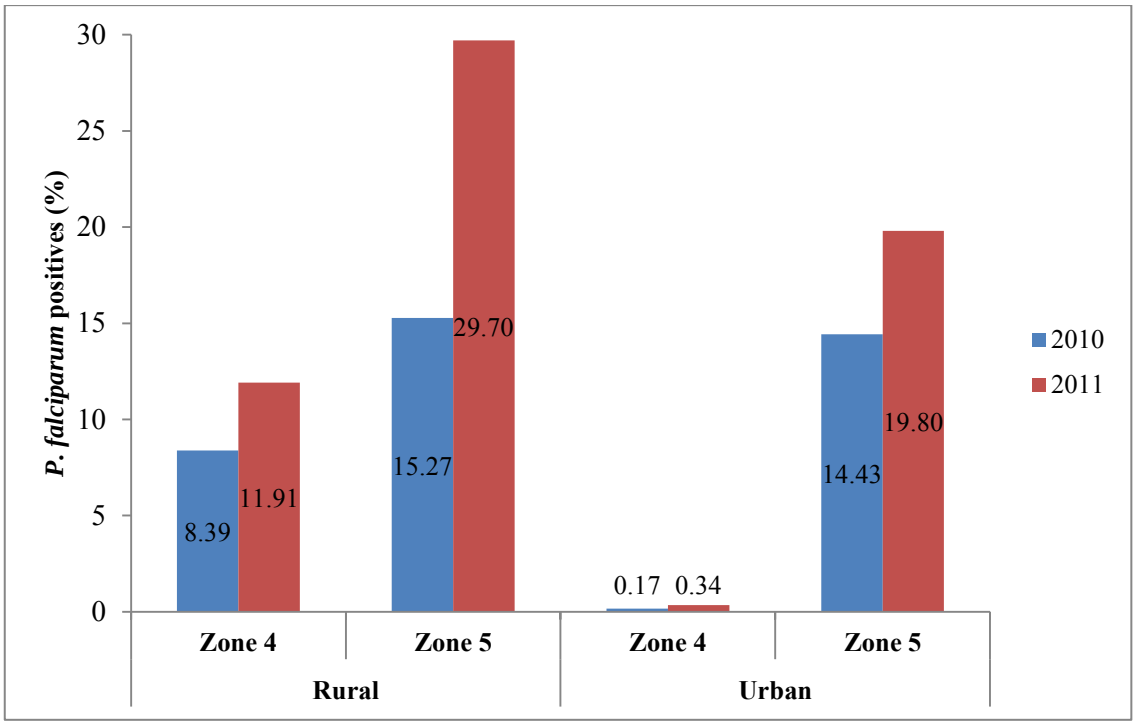


Figure 3.2: Percentage distribution of PCR-confirmed *P. falciparum* positive samples collected in rural and peri-urban sites in Zones 4 and 5 over the study period.

3.2.2. Asexual parasite prevalence

Asexual parasite prevalence was analyzed using q-PCR. An example of the outcome of the analysis is represented by an image in Figure 3.3. The difference in PCR-confirmed asexual parasite prevalence between 2010 and 2011 was examined. In 2010 the observed PCR-confirmed prevalence was 7.80% (228/2924) and in 2011, it was 12.17% (368/3023), not accounting for survey design. However, after logistic regression analysis, this difference held no statistical significance (OR: 0.94; 95% CI: 0.64-1.38; *P*= 0.731) as confirmed by multivariate regression analysis (Table 3.3).

The relation between PCR-confirmed asexual parasite prevalence and study zone was not significant (Pearson Chi-squared test p-value=0.459). There were no significant associations between asexual parasite prevalence and the age of participants (OR: 1.02; 95% CI: 0.96-1.08; *P*=0.541); gender (OR: 1.11; 95% CI: 0.83-1.48; *P*=0.457) or residence in a peri-urban area vs. rural area (OR: 1.52; 95% CI: 0.53-4.34; *P*=0.416) as confirmed by multivariate regression analysis (Table 3.3).

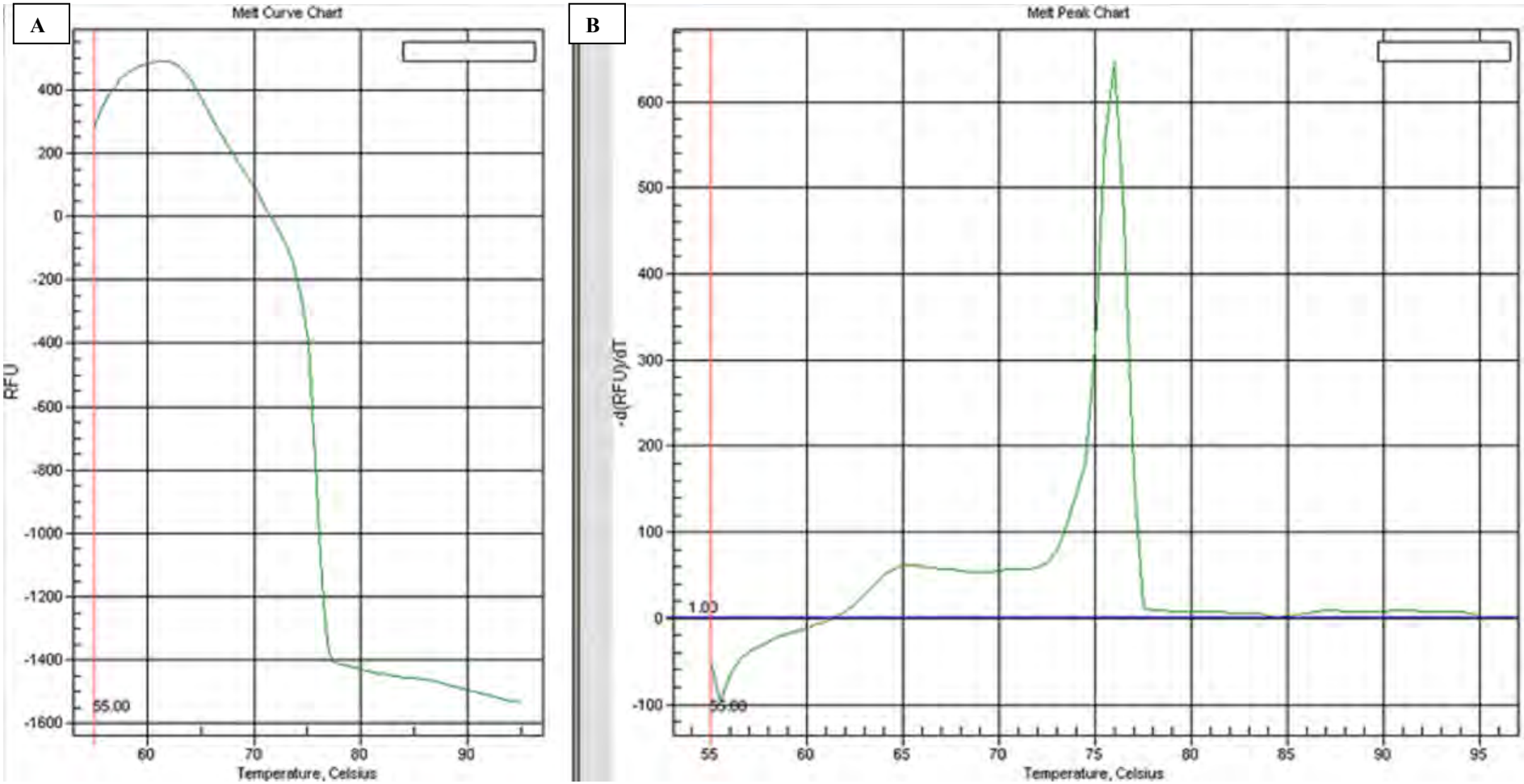


Figure 3.3: Quantitative-PCR amplification with SYBR Green fluorescence detection. A: A quantitative PCR plot obtained for *P. falciparum* identification using the dye FAM. B: Melting curve analysis of the amplification product showing a peak at 76 ºC indicating a *P. falciparum* infection. The presence of one sharp peak shows that there is no contaminating product present in this reaction.

Table 3.3: Output from a multivariate analysis of predefined factors associated with PCR-confirmed asexual parasite prevalence in Zones 4 and 5 between 2010 and 2011

	Asexual parasite prevalence in Zones 4 and 5			Asexual parasite prevalence in Zone 4			Asexual parasite prevalence in Zone 5		
Factor	**OR***	**95% CI****	**p-value**	**OR***	**95% CI****	**p-value**	**OR***	**95% CI****	**p-value**
Year	0.97	0.63-1.51	0.900	1.12	0.56-2.25	0.724	0.85	0.38-1.89	0.656
Age of participant (years)	1.02	0.96-1.08	0.579	1.01	0.90-1.13	0.870	1.03	0.95-1.12	0.412
Residence in a peri-urban area vs. rural area	1.52	0.52-4.42	0.421	0.13	0.09-0.21	<0.001	1.55	0.51-4.78	0.406
Gender	1.09	0.81-1.45	0.564	1.66	1.20-2.28	0.007	0.87	0.60-1.26	0.424

*Odds ratio
**95% confidence interval

The PCR-confirmed asexual parasite prevalence between 2010 and 2011 within Zone 4 changed from 4.46% (51/1144) in 2010 to 6.07% (73/1202) in 2011 (Figure 3.4) not accounting for survey design. After logistic regression analysis, this change was not significant (OR: 1.08; 95% CI: 0.54-2.15; *P*= 0.797). Similarly in Zone 5 the prevalence changed from 9.94% (177/1780) in 2010 to 16.20% (295/ 1821) in 2011 not accounting for survey design. After logistic regression analysis, this change was not significant (OR: 0.811; 95% CI: 0.40-1.64; *P*= 0.526).

In Zone 4, asexual parasite prevalence was positively associated with gender (OR: 1.60; 95% CI: 1.15-2.22; *P*=0.011); and negatively associated with residence in a peri-urban area vs. a rural area (OR: 0.14; 95% CI: 0.09-0.20; *P*<0.001) as confirmed by multivariate regression analysis (Table 3.3). Asexual parasite prevalence in Zone 4 was not significantly associated with the age of participants (OR: 1.01; 95% CI: 0.91-1.12; *P*=0.851). In Zone 5, there were no significant associations between asexual parasite prevalence and the age of participants (OR: 1.03; 95% CI: 0.95-1.12; *P*=0.390); gender (OR: 0.90; 95% CI: 0.62-1.29; *P*=0.524) or residence in a peri-urban area vs. a rural area (OR: 1.56; 95% CI: 0.52-4.69; *P*=0.389) as confirmed by multivariate logistic regression (Table 3.3).

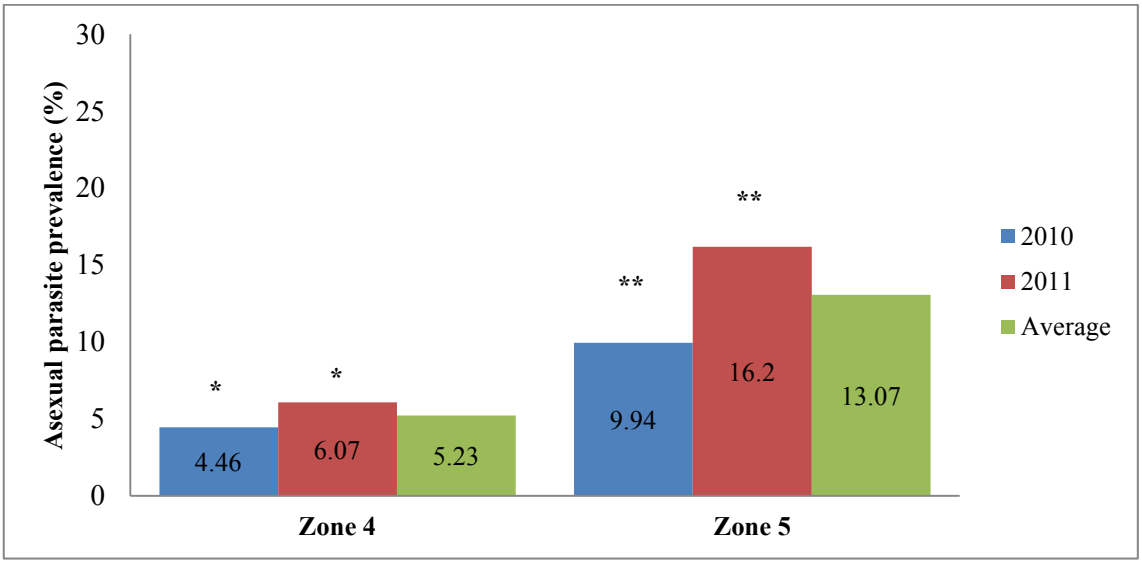


Figure 3.4: Asexual parasite prevalence in Zones 4 and 5 over the study period. The average indicates the change in parasite prevalence over the study period in Zones 4 and 5. **P*=0.797 ** *P*=0.526.

3.2.3. Multiplicity of infection

The multiplicity of infection, which is the number of *P. falciparum* populations present in each sample, was determined by the amplification of the msp-2 molecular marker. An example of the msp-2 marker analysis is shown in Figure 3.5.

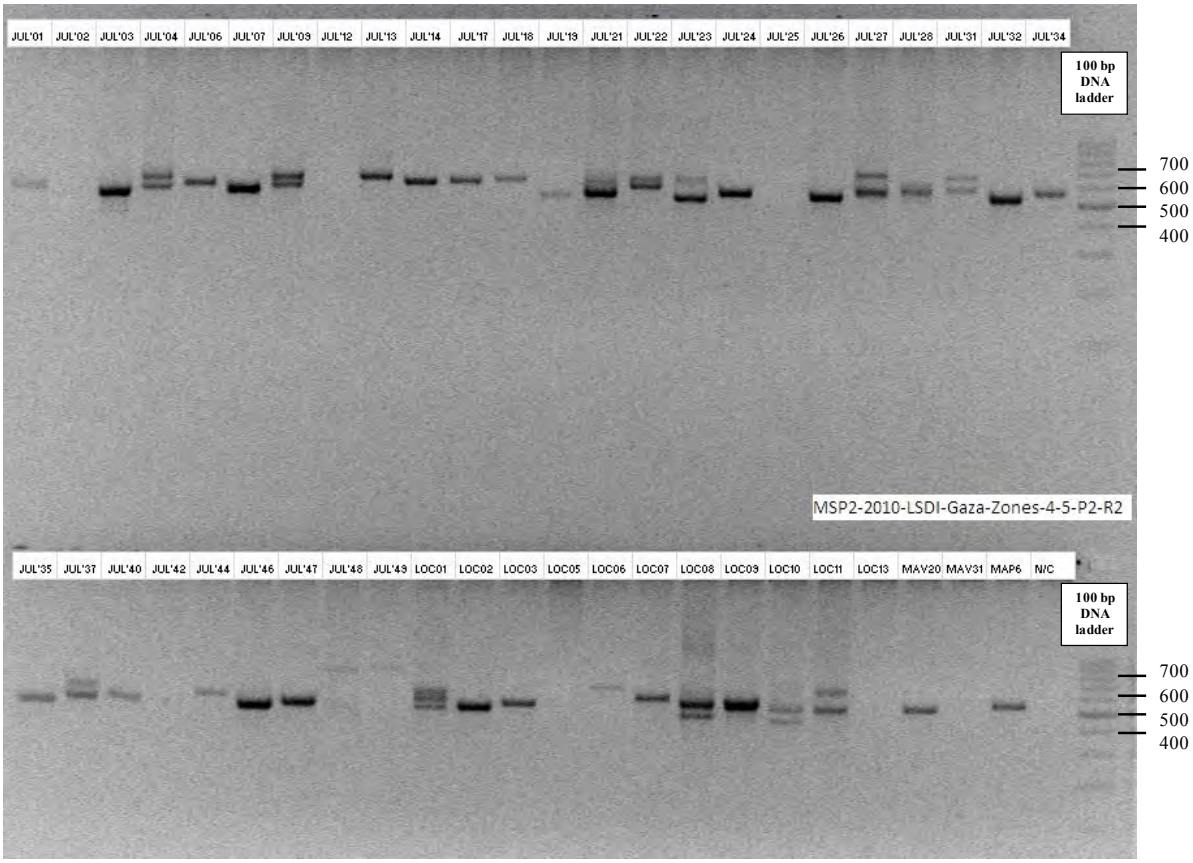


Figure 3.5: An image of a 2% agarose gel showing msp-2 alleles amplified using nested PCR. The labels above each well is the laboratory identity assigned to each sample. One band represents a single *P. falciparum* infection, with 2 and 3 bands representing 2 and 3 genetically distinct populations respectively.

Of 596 PCR-confirmed *P. falciparum* positive samples, 482 (80.87%) were successfully subjected to msp-2 genotyping. The majority of samples genotyped (308/482) contained a single infecting *P. falciparum* population (Table 3.4) with 33.61% carrying two distinct infections and 2.49% carrying three.

In 2010, the prevalence of samples with multiple infecting populations was 26.38% which changed to 41.07% in 2011, not accounting for survey design (Figure 3.6). There was a statistically significant increase in the prevalence of samples with multiple infecting populations after considering survey design in a logistic regression analysis (OR: 1.94; 95% CI: 1.39-2.71; P<0.001). This observation was confirmed by multivariate regression analysis (Table 3.5 p 53). The prevalence of multiple infecting *P. falciparum* populations within a sample was negatively associated with gender (OR: 0.65; 95% CI: 0.43-0.96; P=0.033) though not significantly associated with the age of participants (OR: 0.96; 95% CI: 0.91-1.02; P=0.151) or residence in a peri-urban vs. rural area (OR: 1.32; 95% CI: 0.87-2.00; P=0.186) as confirmed by multivariate regression analysis (Table 3.5 p 53).

Table 3.4: Output from a multivariate analysis of predefined factors associated with PCR-confirmed asexual parasite prevalence in Zones 4 and 5 between 2010 and 2011

No. of infecting *P. falciparum* populations	No. of PCR-confirmed *P. falciparum* samples	% of total samples amplified for msp-2
1	308	63.90
2	162	33.61
3	12	2.49

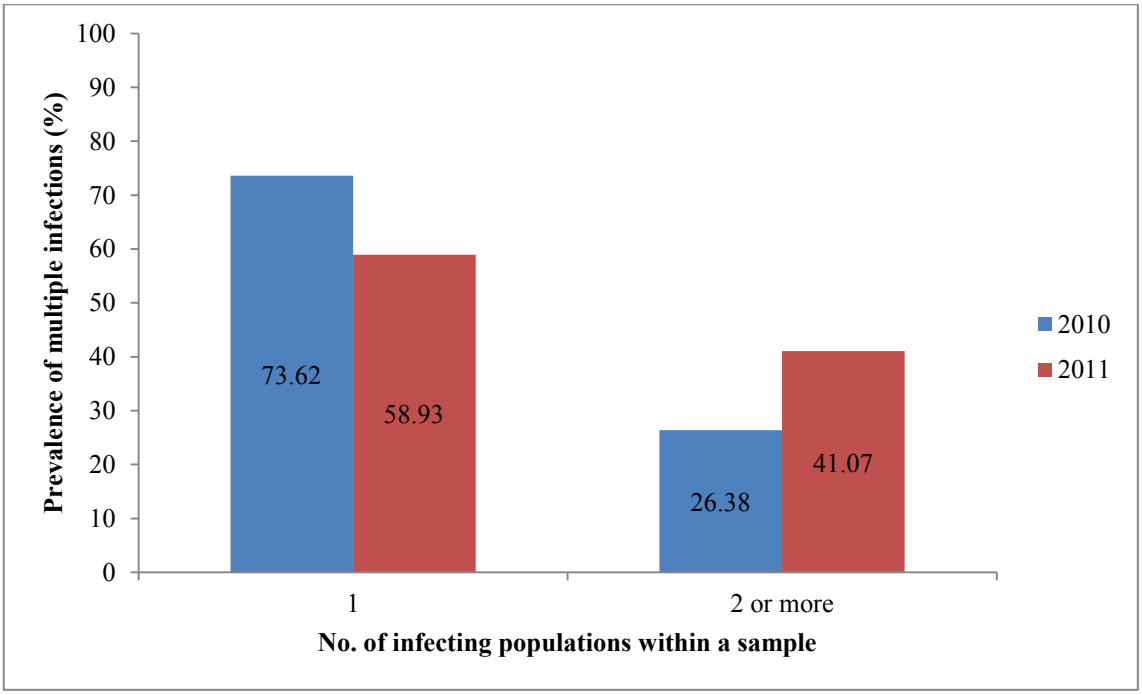


Figure 3.6: The comparison between samples containing one infecting parasite population and genetically diverse *P. falciparum* samples (as determined by the presence of 2 or more populations within single samples) in 2010 and 2011 in the study area.

The multiplicity of infection was assessed per zone. In Zone 4, the prevalence increased from 18.75% (6/32) in 2010 to 25.76% (17/66) in 2011 not accounting for survey design. This increase was not significant when computed in a logistic regression model (OR: 1.50; 95% CI: 0.67-3.38; *P*=0.272) as confirmed by multivariate logistic regression (Table 3.5). However, in Zone 5, the increase from 24.43% (37/131) in 2010 to 45.06% (114/253) in 2011 was significant (OR: 2.08; 95% CI: 1.55-2.81; *P*<0.001) after accounting for survey design. In Zone 4, there were no significant associations between multiplicity of infection and the age of participants (OR: 1.08; 95% CI: 0.95-1.23; *P*=0.202) or gender (OR: 1.12; 95% CI: 0.34-3.67; *P*=0.826) whilst residence was omitted due to co-linearity as confirmed by multivariate logistic regression (Table 3.5). In

Zone 5, negative associations between multiplicity of infection and both the age of participants (OR: 0.94; 95% CI: 0.89-0.99; *P*=0.021) and gender (OR: 0.58; 95% CI: 0.38-0.88; *P*=0.016) were statistically significant whilst an association with residence in a peri-urban area vs. a rural area held no significance (OR: 1.08; 95% CI: 0.77-1.52; *P*=0.634) as confirmed by multivariate logistic regression (Table 3.5).

Table 3.5: Output from a multivariate analysis of predefined factors associated with prevalence of multiplicity of infection in Zones 4 and 5 between 2010 and 2011

	Prevalence of multiplicity of infection			**Prevalence of multiplicity of infection in Zone 4**			**Prevalence of multiplicity of infection in Zone 5**		
Factor	**OR***	**95% CI****	**p-value**	**OR***	**95% CI****	**p-value**	**OR***	**95% CI****	**p-value**
Year	1.97	1.51-2.57	<0.001	0.56	0.73-3.36	0.211	2.07	1.61-2.66	<0.001
Age of participant (years)	0.96	0.92-1.02	0.163	1.09	0.96-1.23	0.149	0.94	0.89-0.98	0.014
Residence in a peri-urban area vs. a rural area	1.39	0.82-2.37	0.210	Omitted***			1.15	0.72-1.83	0.517
Gender	0.65	0.43-0.98	0.042	1.11	0.28-4.38	0.868	0.59	0.38-0.91	0.022

*Odds ratio
**95% confidence interval
***Excluded from the model because of co-linearity

3.2.4. Sulphadoxine-pyrimethamine resistance marker prevalence

The 596 PCR-confirmed *P. falciparum* positives were subjected to *dhfr* 108, 51 and 59, and *dhps* 436, 437, 540 and 581 mutational analyses. Of these, 590 samples were successfully amplified by nested PCR for all molecular markers. An example of the outcome of the mutational analysis is displayed in Figure 3.7 and in the Appendix.

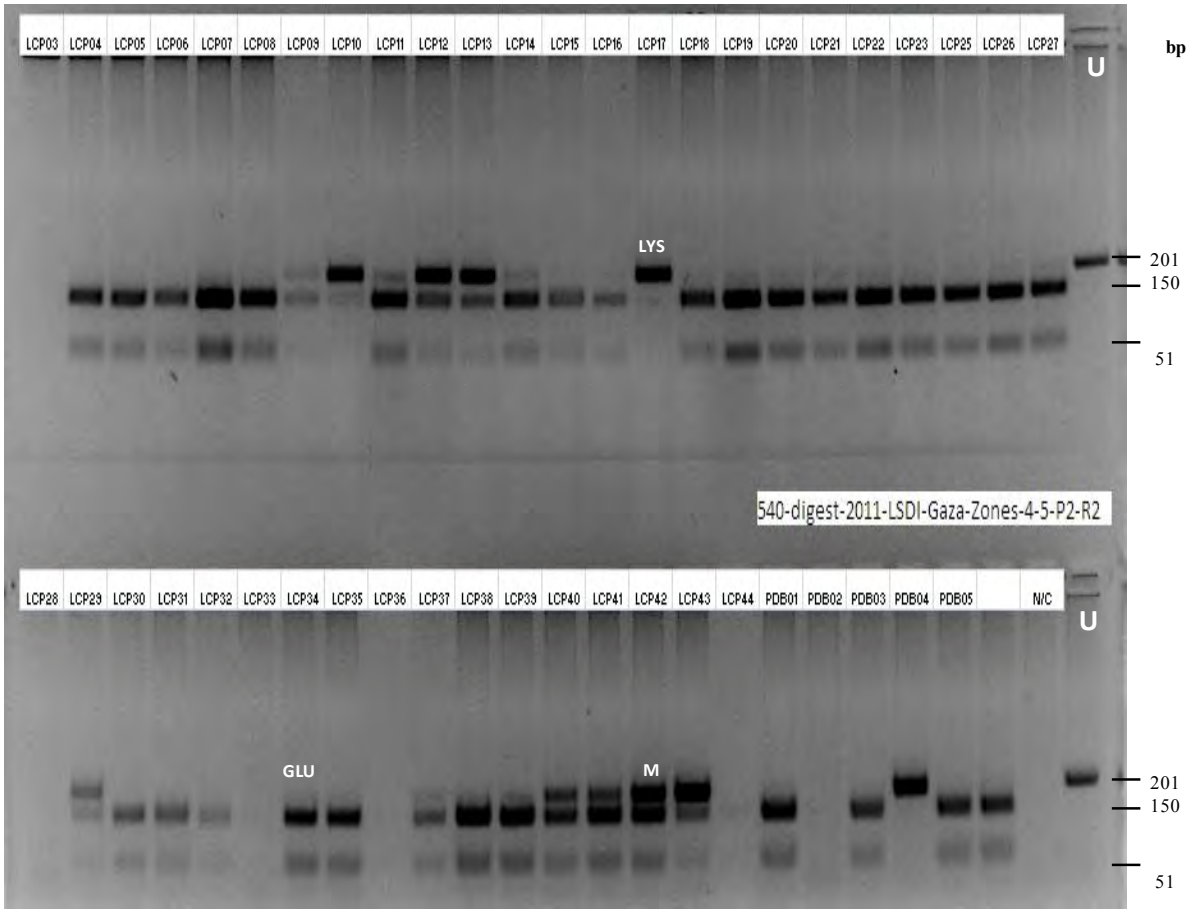


Figure 3.7: An image of a 2% agarose gel showing the restriction fragments obtained following the digestion of amplified products containing the *dhps* 540 allele with the restriction enzyme FokI. The label above each well is the unique laboratory identity assigned to each sample. An undigested PCR amplified DNA fragment (U) containing the *dhps* 540 allele was used as a molecular marker to differentiate between wildtype and mutant alleles. The presence of a single uncut fragment (201 bp) denotes a sensitive genotype, with the amino acid lysine (LYS) at codon 540 in the *dhps* gene. The presence of a smaller fragment (150 bp) denotes the presence of a *dhps* mutant allele with the amino acid glutamic acid (GLU) replacing lysine at *dhps* codon 540. M indicates a sample is carrying two or more *P. falciparum* parasite populations, one of which is wildtype at *dhps* 540 and one mutant at *dhps* 540.

The *dhfr* triple mutation (108N+51I+59R), associated with pyrimethamine resistance, remained at a high prevalence over the study period: 99.11% (223/225) in 2010 and 99.73% (364/365) in 2011 (OR: 3.26; 95% CI: 0.76-14.00; *P*= 0.106) (Figure 3.8) as confirmed by multivariate regression analysis (Table 3.6). Due to the near saturation of the *dhfr* triple mutation across the study area, no association between the *dhfr* triple mutation prevalence and residence could be estimated. Prevalence of this mutation combination was not significantly associated with the age of participants (OR: 0.91; 95% CI: 0.61-1.36; *P*=0.635) or gender (OR: 0.54; 95% CI: 0.26-11.37; *P*=0.678) as confirmed by multivariate regression analysis (Table 3.6).

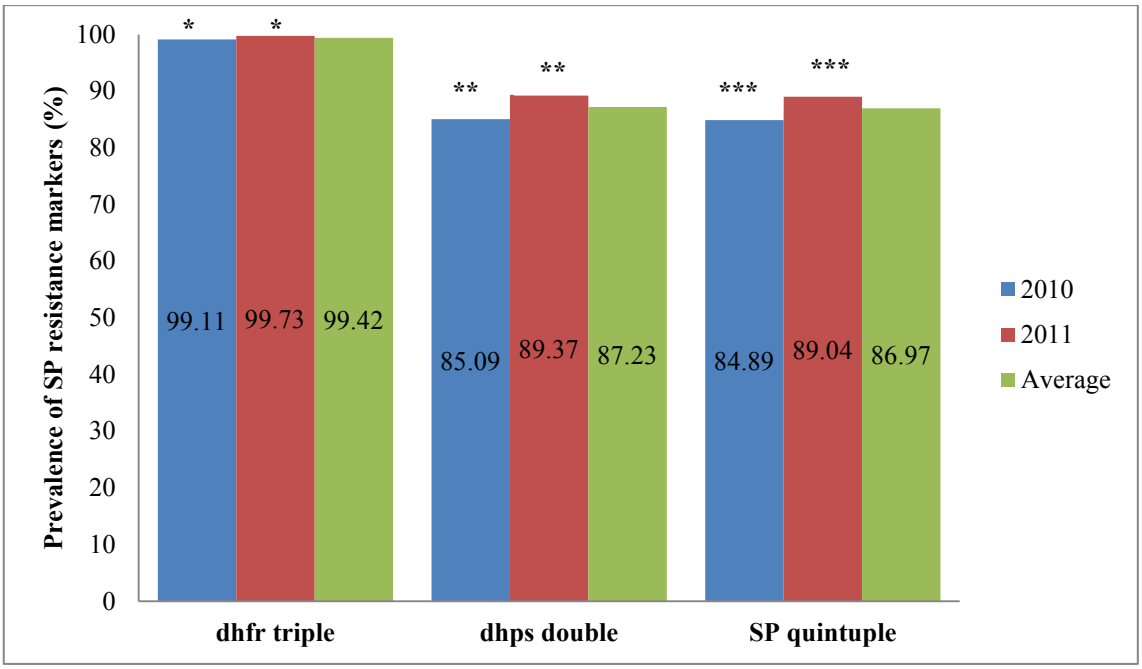


Figure 3.8: Prevalence of the *dhfr* triple, *dhps* double and sulphadoxine-pyrimethamine (SP) quintuple mutations associated with SP resistance in Zones 4 and 5 (combined) in 2010 and 2011. The average indicates the change in parasite prevalence over the study period in Zones 4 and 5. **P*= 0.106 ** *P*=0.293 *** *P*=0.325.

Table 3.6: Output from a multivariate analysis of predefined factors associated with *dhfr* triple, *dhps* double and sulphadoxine-pyrimethamine (SP) quintuple mutation prevalence in Zones 4 and 5 between 2010 and 2011

	***dhfr* triple mutation prevalence**			***dhps* double mutation prevalence**			**SP quintuple mutation prevalence**		
Factor	**OR***	**95% CI****	**p-value**	**OR***	**95% CI****	**p-value**	**OR***	**95% CI****	**p-value**
Year	3.55	0.84-15.03	0.080	1.52	0.75-3.05	0.227	1.48	0.72-3.04	0.264
Age of participant (years)	0.91	0.61-1.36	0.610	1.01	0.93-1.08	0.880	1.00	0.93-1.07	0.972
Residence in a peri-urban area vs. a rural area	Omitted***			1.67	0.70-3.98	0.229	1.71	0.70-4.15	0.223
Gender	0.49	0.02-14.05	0.658	0.73	0.51-1.10	0.091	0.70	0.50-0.98	0.040

*Odds ratio
**95% confidence interval
***excluded from the model because of co-linearity

In 2010, the *dhfr* triple mutation prevalence was 96.08% (49/51) in Zone 4 (Figure 3.9) and 100 % (174/174) in Zone 5 (Figure 3.10). In 2011, the *dhfr* triple mutation prevalence increased to 98.63% (72/73) in Zone 4 (OR: 2.94; 95% CI: 0.75-11.52; *P*= 0.106) (Figure 3.9) though this was not statistically significant, and remained at 100% (293/293) in Zone 5 (Figure 3.10). In Zone 4, the prevalence of the *dhfr* triple mutation was not significantly associated with the age of participants (OR: 0.94; 95% CI: 0.60-1.49; *P*=0.778) or gender (OR: 0.56; 95% CI: 0.02-2.59; *P*=0.722) as confirmed by multivariate regression analysis (Table 3.7). The association between *dhfr* triple mutation prevalence and residence could not be assessed due to co-linearity. Due to the fixation of the *dhfr* triple mutation in Zone 5, the associations between *dhfr* triple mutation prevalence and any of the factors could not be performed. Despite the fixation of the highly resistant *dhfr* triple mutation, the *dhfr* I164L mutation, associated with higher levels of pyrimethamine resistance, was absent in all the parasite isolates tested. Mixed alleles at codons *dhfr*51, *dhfr*59 or *dhfr*108 were extremely rare, being detected in 0.8% (5/596) of the samples analysed.

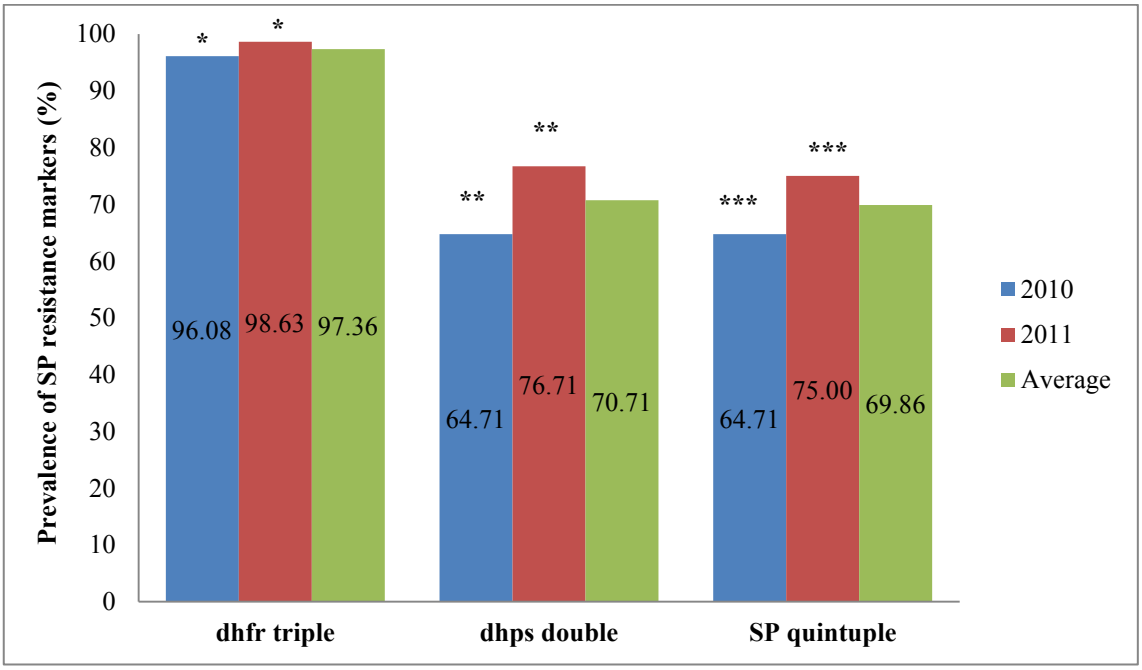


Figure 3.9: Prevalence of the *dhfr* triple, *dhps* double and sulphadoxine-pyrimethamine (SP) quintuple mutations associated with SP resistance in Zone 4 in 2010 and 2011. The average indicates the change in parasite prevalence over the study period in Zones 4 and 5. **P*= 0.106 ***P*=0.207 *** *P*=0.298.

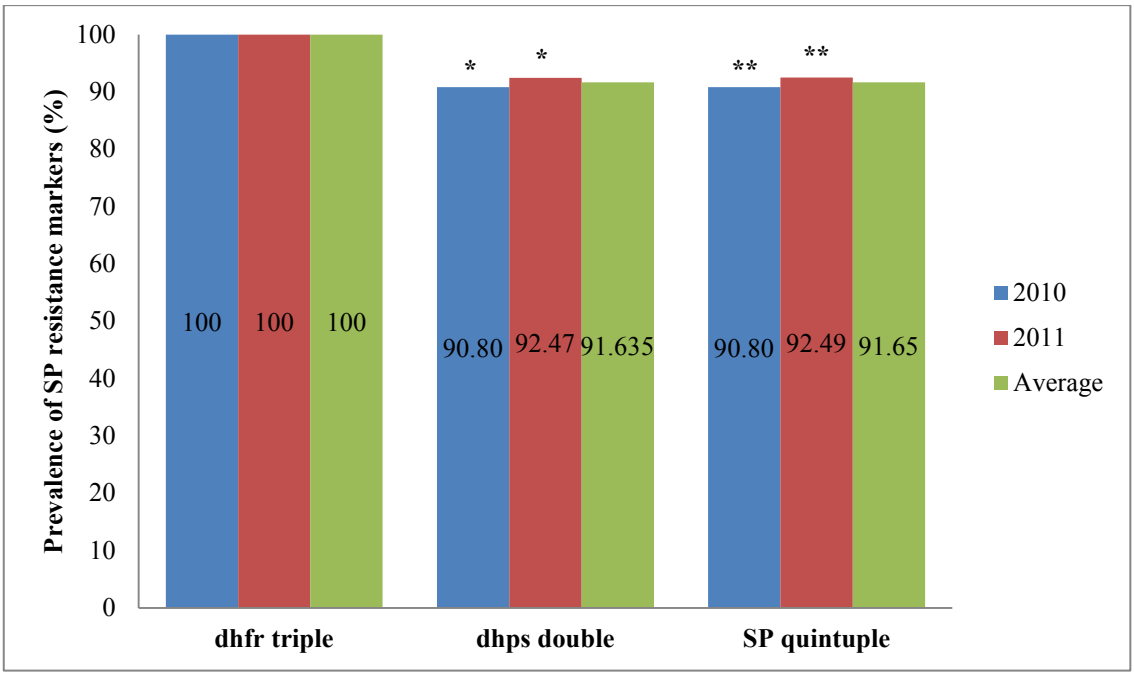


Figure 3.10: Prevalence of the *dhfr* triple, *dhps* double and sulphadoxine-pyrimethamine (SP) quintuple mutations associated with SP resistance in Zone 5 in 2010 and 2011. The average indicates the change in parasite prevalence over the study period in Zones 4 and 5. **P*=0.640 ** *P*=0.616.

Table 3.7: Output from a multivariate analysis of predefined factors associated with *dhfr* triple, *dhps* double and sulphadoxine-pyrimethamine (SP) quintuple mutations prevalence in Zone 4 between 2010 and 2011

	***dhfr* triple mutation prevalence**			***dhps* double mutation prevalence**			**SP quintuple mutation prevalence**		
Factor	**OR***	**95% CI****	**p-value**	**OR***	**95% CI****	**p-value**	**OR***	**95% CI****	**p-value**
Study year	2.77	0.48-15.87	0.210	1.85	0.67-5.10	0.198	1.67	0.57-4.89	0.305
Age of participant (years)	0.94	0.58-1.53	0.767	1.02	0.88-1.17	0.794	1.01	0.88-1.16	0.900
Residence in a peri-urban area vs. a rural area	Omitted***			0.74	0.29-1.87	0.476	0.76	0.30-1.93	0.519
Gender	0.57	0.01-31.68	0.748	0.80	0.45-1.45	0.420	0.73	0.43-1.24	0.211

*Odds ratio

**95% confidence interval
***excluded from the model because of co-linearity

In 2010, the *dhps* double mutation prevalence was approaching fixation, with 85.09% (194/228) of all the parasite extracts analysed carrying the *dhps* 437G and *dhps* 540E mutation alleles (Figure 3.8 p 55). By 2011, the prevalence of this double mutation had increased to 89.37% (328/367) (Figure 3.8 p 55) not accounting for survey design. However, this increase was not statistically significant (OR: 1.47; 95% CI: 0.70-3.12; *P*=0.293) when computed in a logistic regression model as confirmed by multivariate regression analysis (Table 3.6 p 55). The prevalence of the *dhps* double mutation was not significantly associated with the age of participants (OR: 1.01; 95% CI: 0.94-1.09; *P*=0.798); gender (OR: 0.74; 95% CI: 0.51-1.07; *P*=0.102) or residence in a peri-urban area vs. a rural area (OR: 1.59; 95% CI: 0.65-3.89; *P*=0.294) as confirmed by multivariate regression analysis (Table 3.6 p 55).

Although the *dhps* double mutation prevalence increased from 64.71% (33/51) to 76.71% (56/73) over the study period in Zone 4, when not accounting for survey design, this increase was not statistically significant (OR: 1.80; 95% CI: 0.66-4.86; *P*=0.207) (Figure 3.9 p 56) after logistic regression analysis. Similarly, in Zone 5, the increase in *dhps* double mutation prevalence from 90.80% (158/174) in 2010 to 92.47% (270/292) in 2011 before logistic regression analysis held no statistical significance (OR: 1.23; 95% CI: 0.48-3.16; *P*=0.640) (Figure 3.10) once computed in the regression model. None of the other factors were significantly associated with *dhps* double mutation prevalence in Zone 4: the age of participants (OR: 1.02; 95% CI: 0.87-1.21; *P*=0.748); gender (OR: 0.80; 95% CI: 0.42-1.55; *P*=0.456) or residence in a peri-urban area vs. a rural area (OR: 0.78; 95% CI: 0.39-1.57; *P*=0.433) as confirmed by multivariate regression analysis (Table 3.7). Similarly, there were no significant associations in Zone 5: the age of participants (OR: 1.01; 95% CI: 0.92-1.12; *P*=0.764); gender (OR: 0.69; 95% CI: 0.41-1.16; *P*=0.141) or residence in a peri-urban area vs. a rural area (OR: 0.83; 95% CI: 0.29-2.34; *P*=0.698) as confirmed by multivariate regression analysis (Table 3.8). Mutant alleles at codons *dhps* 436 and *dhps* 581 were not detected in any of the samples analysed during the study period. Mixed alleles at *dhps* codons 437 and 540 were detected in 17.4% (104/596) of the samples analysed.

Table 3.8: Output from a multivariate analysis of predefined factors associated with *dhps* double and sulphadoxine-pyrimethamine (SP) quintuple mutation prevalence in Zone 5 between 2010 and 2011

	***dhps* double mutation prevalence**			**SP quintuple mutation prevalence**		
Factor	**OR***	**95% CI****	**p-value**	**OR***	**95% CI****	**p-value**
Study year	1.21	0.45-3.27	0.676	1.22	0.45-3.28	0.670
Age of participant (years)	1.02	0.92-1.12	0.751	1.01	0.92-1.12	0.761
Residence in a peri-urban area vs. a rural area	0.86	0.29-2.53	0.768	0.86	0.29-2.53	0.763
Gender	0.69	0.40-1.17	0.149	0.69	0.41-1.17	0.152

*Odds ratio
**95% confidence interval

As the *dhfr* triple mutation was approaching fixation, the prevalence of the SP quintuple mutation was similar to that of the *dhps* double mutation increasing from 84.89% (191/225) in 2010 to 89.04% (325/365) by 2011 not accounting for survey design. However, once the logistic regression model was run, this increase showed no statistical significance (OR: 1.45; 95% CI: 0.67-3.11; *P*=0.325) (Figure 3.8 p 55) as confirmed by multivariate regression analysis (Table 3.6 p 55). Gender was significantly negatively associated with SP quintuple mutation prevalence (OR: 0.71; 95% CI: 0.50-1.00; *P*= 0.049) whilst the age of participants (OR: 1.01; 95% CI: 0.94-1.08; *P*= 0.880) and residence in a peri-urban area vs. a rural area (OR: 1.62; 95% CI: 0.65-4.10; *P*=0.287) did not appear to influence the SP quintuple mutation prevalence across the study area as confirmed by multivariate regression analysis (Table 3.6 p 55).

The SP quintuple mutation prevalence increased from 64.71% (33/51) to 75% (54/72) in Zone 4 (OR: 1.64: 95% CI: 0.58-4.61; *P*=0.298) (Figure 3.9 p 56) and from 90.80% (158/174) to 92.49% (271/293) in Zone 5 (OR: 1.25; 95% CI: 0.49-3.21; *P*=0.616) (Figure 3.10 p 57) over the study period when not accounting for survey design but these increases held no statistical significance after computed in a logistic regression model as confirmed by multivariate regression analysis (Tables 3.7 and 3.8). None of the factors tested in a logistic variable model were significantly associated with SP quintuple mutation prevalence in Zone 4: the age of participants (OR: 1.01;

95% CI: 0.86-1.20; *P*=0.836); gender (OR: 0.73; 95% CI: 0.41-1.31; *P*=0.243) or residence in a peri-urban area vs. a rural area (OR: 0.82; 95% CI: 0.39-1.75; *P*=0.563) as confirmed by multivariate regression analysis (Table 3.7). Similarly, there were no significant associations in Zone 5: the age of participants (OR: 1.01; 95% CI: 0.92-1.12; *P*=0.769); gender (OR: 0.68; 95% CI: 0.41-1.14; *P*=0.128) or residence in a peri-urban area vs. a rural area (OR: 0.83; 95% CI: 0.29-2.35; *P*=0.696) as confirmed by multiple regression analysis (Table 3.8). The association of multiplicity of infection (2 or more infections) with sulphadoxine-pyrimethamine resistance held no significance (OR: 1.57; 95% CI: 0.80-3.06; *P*=0.174) in the study area as in Zone 4 (OR: 0.73; 95% CI: 0.16-3.48; *P*=0.645) though a statistically significant positive association was observed in Zone 5 (OR: 1.83; 95% CI: 1.11-3.01; *P*=0.021).

3.3. Discussion

3.3.1. Asexual parasite prevalence in Zones 4 and 5

In this study, asexual parasite prevalence was assessed by the use of RDTs in the field, the results of which were confirmed by PCR in the laboratory. While microscopy remains the gold standard for malaria diagnosis (Rakotonirina *et al*., 2008) and would have been another accurate measure of asexual parasite prevalence, microscopy in field conditions would have not been feasible in this study. Rapid diagnostic tests, the type which detects the HRP-2 antigen in the blood, were preferred over microscopy due to their relative ease of transport, fast results and user-friendliness of the test (Ishengoma *et al*., 2011). Rapid diagnostic tests are suitable for their purely qualitative assessment of the patient blood samples which was suitable for this cross-sectional study (Wongsrichanalai *et al*., 2007). Laboratory analysis of filter paper blood spots using a nested PCR method revealed an over-reporting of the number of *P. falciparum* positive samples collected in the field. This is probably because the histidine-rich protein 2, a malaria diagnostic indicator used in the RDTS, remains in the peripheral blood for a period of time after the infection has been cleared giving a false positive RDT result (Hendriksen *et al*., 2011). The PCR-confirmed asexual parasite prevalence was therefore used as the indicator of parasite prevalence in this study.

The PCR-confirmed asexual parasite prevalence in the study area remained at low levels. This is not surprising considering that integrated malaria control measures have been in place since 2006 as part of the malaria control arm of the Lubombo Spatial Development Initiative (Lubombo Spatial Development Initiative, 2010). Vector control by indoor residual spraying and effective case management by antimalarial diagnosis and treatment, two arms of the initiative, have been integral in lowering the prevalence of malaria in Gaza Province and neighbouring Maputo Province (Lubombo Spatial Development Initiative, 2010).

3.3.2. Prevalence of sulphadoxine-pyrimethamine resistance markers in Zones 4 and 5

High levels of markers associated with sulphadoxine-pyrimethamine resistance were observed in the study in line with a previous research study conducted in the region (Lubombo Spatial Development Initiative, 2010). Despite the change from artesunate plus sulphadoxine-pyrimethamine to artemether-lumefantrine for first-line treatment of uncomplicated *P. falciparum* malaria in Mozambique in 2008, sulphadoxine-pyrimethamine resistance marker remains at high levels in the study population, contrasting previous reports of observed decreases in antimalarial drug resistance once a drug has been discontinued as observed with chloroquine resistance markers in Malawi (Kublin *et al.*, 2003). While resistant parasites have a 7-13% fitness disadvantage relative to sensitive parasites in the absence of drug pressure the withdrawal of drugs from circulation and subsequent removal of drug pressure does not always result in the re-emergence of drug-sensitive parasite strains (Anderson and Roper, 2005).

Sulphadoxine-pyrimethamine resistance marker prevalence could be maintained by secondary, compensatory mutations that are present in other regions of the parasite genome (Hastings and Donnelly, 2005). These compensatory mutations may provide a survival advantage to the resistant parasites over the sensitive ones in the absence of drug pressure (Hastings and Donnelly, 2005). The mechanisms involved could affect survival fitness, replication and transmission probability, invasion, reproduction and vector properties that favour transmission (Marks *et al*., 2005). Mutations conferring drug resistance may arise frequently and its transmission may be affected by compensatory mutations that restore parasite fitness (Anderson and Roper, 2005). However, the probability of both drug resistance and compensatory mutations in the parasite genome is small and compensatory mutations in *P. falciparum* have not yet been identified (Anderson and Roper, 2005).

Another possibility for the survival of resistant parasites in the absence of drug pressure is that if resistant strains are highly prevalent in the population, almost to a point of fixation, there may not be sufficient numbers of sensitive parasites to compete with resistant ones resulting in a fixation of resistance in the population (Raman *et al*., 2008). This is a possibility in Gaza Province as the SP quintuple mutation is at extremely high prevalence in the population.

The current SP quintuple mutation prevalence in Gaza Province could also be influenced by sustained sulphadoxine-pyrimethamine drug pressure in the region through the use, since 2007, of sulphadoxine-pyrimethamine monotherapy for intermittent preventive treatment in pregnant women (Allen, *et al*., 2009; Raman *et al*., 2010). The World Health Organization (WHO) advises that sulphadoxine-pyrimethamine monotherapy for intermittent preventive treatment could still be

effective in areas where sulphadoxine-pyrimethamine efficacy in children is 50% (World Health Organization, 2010a). There have been reports however, that sulphadoxine-pyrimethamine for intermittent preventive treatment loses its efficacy when there is a high level of sulphadoxine-pyrimethamine resistance (Harrington *et al*., 2011). Given the high levels of the SP quintuple mutation prevalence reported in this study, this form of prophylaxis with sulphadoxine-pyrimethamine could be severely compromised and therefore needs to be reconsidered.

Yet another factor that may contribute to sulphadoxine-pyrimethamine drug pressure in the region is the use of trimethroprim-sulfamethoxazole (co-trimoxazole) as a treatment for bacterial infections in HIV/AIDS patients. Co-trimoxazole is an antifolate combination that has similar mechanisms of action to sulphadoxine-pyrimethamine, which can therefore sustain antifolate drug pressure and confer cross-resistance (Brentlinger *et al*., 2006; Thera *et al.*, 2005). Given the high prevalence of HIV/AIDS infections in Mozambique and the increased HIV/AIDS treatment delivery undertaken in the country, co-trimoxazole, which has been administered to the majority of the population (Audet *et al*., 2010) could possibly provide sufficient drug pressure to maintain antifolate resistance (Laufer and Plowe, 2004; Marks *et al*., 2005; World Health Organization, 2011b).

The findings of the present study are in line with previously observed prevalence of *dhfr* triple and *dhps* double mutation haplotypes in southern Africa (Enosse *et al*., 2008; Mita *et al*., 2009; Pearce *et al*., 2009; Roper *et al.*, 2003). Drug resistant parasites can spread over large geographical areas via selective sweeps regardless of transmission intensity which is why similar mutation haplotypes occur in different geographic areas (McCollum *et al*., 2008; Mita *et al*., 2009; Pearce *et al*., 2005; Walliker, 2005). Previous studies have shown that Maputo Province, which neighbours Gaza Province, harbours a high prevalence of the *dhfr* triple, *dhps* double and SP quintuple mutation haplotypes with the latter reaching 75% in 2008 (Raman *et al*., 2008; Raman *et al*., 2010). The spread of *P. falciparum* resistance polymorphisms from Maputo Province to Gaza Province is a possible reason for the high levels of sulphadoxine-pyrimethamine resistance observed in this study. The *dhfr* 164L mutation which confers high levels of sulphadoxine-pyrimethamine resistance, has been reported in Southeast Asia (Hyde, 2008) and in Africa, it has been identified in Malawi and Kenya (Alker *et al*., 2005; Maharakurwa *et al*., 2011). The *dhfr* 164L mutation has not been observed in Gaza Province, despite the fixation of the SP quintuple mutation and the presence of the 164L mutation in neighbouring Malawi (Alker *et al*., 2005) and may be attributed to a fitness cost associated with the *dhfr* (108N+51I+ 59R+164L) quadruple mutation (Lozovsky *et al*., 2009).

3.3.3. Prevalence of infections containing multiple parasites

In this study, multiple infections were observed with three being the highest number of infecting parasite populations within a single sample. Multiplicity of infection can be used as an indicator of transmission intensity and immune status with the multiplicity of infection increasing as immunity develops (Males *et al.*, 2008; Vafa *et al.*, 2008). Mixed infections occur due to hosts becoming infected simultaneously (co-infection) or sequentially (superinfection) (de Roode *et al*., 2005). Co-infection is possible because one mosquito may contain different parasite populations whilst superinfection occurs when hosts are bitten numerous times by different mosquitoes (de Roode, *et al*., 2005). Competition can reduce the parasite burden as parasites compete for red blood cells and evasion from immune responses (de Roode *et al*., 2005). Co-infections and superinfections can aid the spread of drug resistance (de Roode, *et al*., 2005). In the presence of drug pressure, drug resistant parasites survive whilst sensitive parasites do not, and this reduces the limits set by competition, known as competitive release, allowing the resistant parasites to increase dramatically and spread faster (de Roode *et al*., 2005). Multiplicity of infection, which increased significantly in this study, enhances the longevity of an infection and gametocyte production potential and is also influenced by seasonal transmission (Nassir *et al.*, 2005; Vafa *et al.*, 2008).

The low prevalence of mixed infections observed in the study suggests that competition within the host between wildtype and mutant populations is intense with mutant parasites effectively eliminating wildtype parasites at the population level (Shah *et al*., 2011). Since only the msp-2 marker method for determining genetic diversity of an infection was used in this study and since the analysis of multiple loci has been reported to improve the detection of multiplicity of infection (Farnert *et al*., 2001; Snounou *et al*., 1999), analysis of the msp-2 marker alone may not reflect the true *P. falciparum* genetic diversity in the population (Cattamanchi *et al*., 2003; Farnert *et al*., 2001; Snounou *et al*., 1999). Transmission intensity plays an indirect role in drug resistance evolution in a population, probably acting through clone multiplicity (Hastings and Watkins, 2005). This possible misrepresentation of mixed infections seems likely given the high number of SP resistant mutations in the population since mixed infections are more likely to occur in drug resistant parasites (Barnes and White, 2005; Talisuna *et al*., 2007).

3.3.4. Associations between study factors and asexual parasite prevalence, sulphadoxine-pyrimethamine resistance prevalence and multiplicity of infection

Multiplicity of infection was found to be associated with gender in this study, with males hosting a higher number of *P. falciparum* populations than woman. Sex hormones are said to influence differences in parasite densities between male and female participants (Males *et al.*, 2008). Parasite

densities were not assessed in this study, though given the association found between multiplicity of infection and gender, it would be of interest to determine if an association exists between multiplicity of infection and sex hormones. Testosterone has been shown to increase susceptibility to the disease in murine models and in humans; a male bias with regards to the disease has been proposed (Pathak *et al*., 2012). Other factors such as an increased male exposure to vectors during night when feeding occurs, treatment seeking behaviour differences between males and females, and the consumption of "vector attracting" substances such as alcohol and tobacco could influence this result (Pathak *et al*., 2012).

Whilst residence in a peri-urban area was not associated with asexual parasite prevalence or sulphadoxine-pyrimethamine resistance, significant associations between multiplicity of infections and residence in Zone 5, which contains more peri-urban areas, has been observed. This suggests that there is a higher likelihood of malaria infection if one resides in a peri-urban area. Mozambique is one of the less developed countries in the world and despite 63% of the population still living in rural conditions, there is a move towards more developed infrastructure, higher social expenditures and a greater employment rate (World Bank, 2012). The rapid development of better infrastructure, tarred roads and improved water drainage systems were personally observed when conducting field surveys; with major developmental differences observed in 2011 compared to 2010. This move towards urbanization, while contributing to the country's growing gross domestic product (World Bank, 2012) may not favour efforts to control prevalence of malaria in the country. In sub-Saharan Africa the cost of urbanization is high resulting in a lack of maintenance of proper sanitation and poor housing management (Keiser *et al*., 2004). As a result, surface water is not drained, creating breeding grounds for vectors which may contribute to the transmission of malaria (Keiser *et al*., 2004). Individuals in urban areas also lack immunity and vectors have adapted to urban environments (Keiser *et al*., 2004). This may be because urban environments differ in their levels of development and well developed areas are usually surrounded by semi-rural ones (Keiser *et al*., 2004). The association between asexual parasite prevalence and residence in a peri-urban area observed in this study highlights the need for malaria control to be adjusted to suit this particular urban setting and environment. This is a reminder that environmental management is still important in controlling the disease especially with the varied levels of development seen in urban areas in large geographical regions (Keiser *et al*., 2004). Steps to ensure proper environmental management could include drainage of swamps, larviciding and house screening (Keiser *et al*., 2004).

The lack of association between the age of participants and asexual parasite prevalence in this study contrasts reports from a recent study conducted in the area where a positive association between asexual parasite prevalence and the age of participants was observed (Raman *et al*., 2011).

This is possibly due to a sampling bias that occurred in this study. It was discovered that at some sites, only school children between the ages of six and ten were surveyed. This skewed sampling occurred as a result of poor weather conditions and poor response to survey participation requests.

3.3.5. Conclusion

In conclusion, although asexual parasite prevalence is still relatively low in Gaza Province, the molecular markers associated with sulphadoxine-pyrimethamine resistance are highly prevalent. This is despite discontinuation of the drug in Gaza Province in 2009 and supports the policy change from artesunate plus sulphadoxine-pyrimethamine to artemether-lumefantrine.

CHAPTER 4: Chloroquine and artemether-lumefantrine resistance marker prevalence in Gaza Province

4.1. Introduction

Chloroquine has been used for the treatment of malaria in Africa since the 1940s (Nuhawa, 2001; Trape, 2001). Though chloroquine was affordable, emergence of chloroquine resistance in the continent in the late 1970s hindered the use of the drug as a first-line antimalarial treatment (Burgess *et al*., 2010; Kremsner and Krishna, 2004; Payne, 1987). The spread of resistance to monotherapies, such as chloroquine, led to the adoption of artemisinin-based combination therapies (ACTs) for the treatment of uncomplicated malaria as recommended by the World Health Organization (WHO) in 2003 (World Health Organization, 2009). In line with this recommendation, chloroquine was replaced with sulphadoxine-pyrimethamine plus amodiaquine in Gaza Province in 2004 which in turn was replaced by the artemisinin-based combination therapy (ACT) artesunate plus sulphadoxine-pyrimethamine in 2006 (Fernandes *et al*., 2007; Raman *et al*., 2011). However, resistance to sulphadoxine-pyrimethamine, as indicated by the prevalence of molecular markers of resistance, increased markedly in Gaza Province prompting a treatment policy change to artemether-lumefantrine for first-line treatment of uncomplicated malaria (Raman *et al*., 2011).

The molecular basis of chloroquine resistance has been proposed to be due to specific mutations in the chloroquine resistance transporter (*pfcrt*) and multidrug resistance (*pfmdr1*) genes. The *pfcrt* gene encodes the PfCRT membrane protein while the *pfmdr1* gene codes for the p-glycoprotein homologue 1 transporter protein (Burgess *et al*., 2010; Su *et al*., 1997; Valderramos and Fidock, 2006). Mutations in these genes are thought to alter the configuration of the transmembrane channels which in turn affects the movement of chloroquine within the parasite (Chaijaroenkul *et al*., 2011). The *pfcrt* 76T mutation is said to be the principle determinant of chloroquine resistance whilst polymorphisms in the *pfmdr1* gene play a modulatory role (Chavchich *et al*., 2010; Lakshmanan *et al*., 2005; Valderramos and Fidock, 2006; Valderramos *et al*., 2010).

The genetic determinants of artemisinin resistance have yet to be elucidated despite the phenotype of slow parasite clearance being strongly associated with artemisinin resistance (Dondorp *et al*., 2009; World Health Organization, 2011c). While a molecular marker is sought, ACT efficacy is currently being assessed by determining the effectiveness of the non-artemisinin partner drug. Polymorphisms in the *pfmdr1* gene have also been associated with increased resistance to lumefantrine, mefloquine, quinine, chloroquine and artemisinins (Dokomajilar *et al*., 2006; Laufer

and Plowe, 2004; White, 2004). Copy number amplification in the *pfmdr1* gene, together with the molecular markers associated with lumefantrine resistance such as the *pfmdr1* N86Y polymorphism, have been shown to be useful predictors of resistance to the lumefantrine (Dokomajilar *et al*., 2006; Ngasala *et al*., 2011; Sisowath *et al*.,2009).

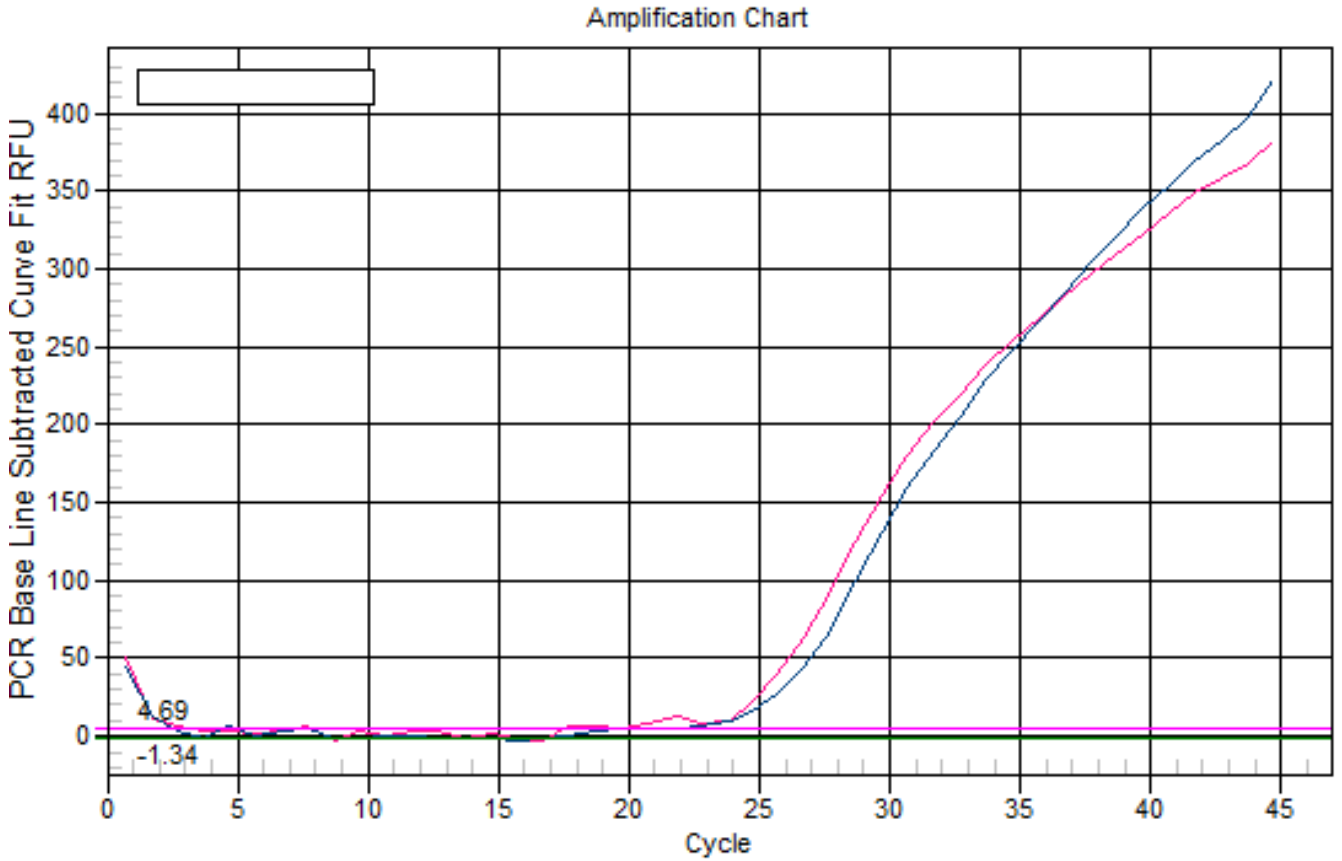
In this chapter, the prevalence of molecular markers associated with chloroquine and lumefantrine resistance in malaria parasite isolates from Gaza Province will be discussed in the context of changes in regional drug pressure. For this study, the presence of the *pfmdr1* N86Y mutation, *pfcrt* CVMNK (wildtype) and *pfcrt* CVIET (mutant) haplotypes as well as *pfmdr1* copy number amplification were assessed. Their associations with prospectively defined malaria risk factors including the age of participants, gender (female vs. male), febrility (temperatures of 37.5 ºC and above vs. those at 37.4 ºC and below) and residence (in a peri-urban area vs. rural area) were examined.

4.2. Results

4.2.1. *pfcrt* resistance marker prevalence

Of the 596 samples confirmed as *P. falciparum* positive by PCR, the *pfcrt* CVMNK/CVIET haplotype associated with chloroquine resistance/sensitivity was amplified by q-PCR in 457 (76.68%) samples. An example of the q-PCR analysis outcome is represented by an image in Figure 4.1. In 2010, the prevalence of the mutant *pfcrt* CVIET haplotype across both Zones 4 and 5 was 34.28% (60/175) increasing to 46.10% (130/282) by 2011. Univariate analysis showed this increase to not be statistically significant (OR: 1.64; 95% CI: 0.58-4.61; *P*=0.330), which was confirmed by multivariate regression analysis (Table 4.1). No association between the *pfcrt* CVIET haplotype prevalence and any of the other factors was found with both univariate: the age of participants (OR: 1.63; 95% CI: 0.58-4.56; *P*=0.333); gender (OR: 0.99; 95% CI: 0.69-1.43; *P*=0.973) or residence in a peri-urban area vs. a rural area (OR: 0.93; 95% CI: 0.46-1.89; *P*=0.833), and multivariate regression analysis (Table 4.1).

PCR Amp/Cycle Chart



Standard Curve Chart

Figure 4.1: Quantitative-PCR amplification of the *pfcrt* 76 gene showing a mixed infection using FAM (pink) and HEX (blue) fluorescent dyes.

Table 4.1: Output from a multivariate analysis of predefined factors associated with the *pfcrt* CVIET haplotype prevalence in Zones 4 and 5 between 2010 and 2011

	***pfcrt* CVIET haplotype prevalence**			***pfcrt* CVIET haplotype prevalence in Zone 4**			***pfcrt* CVIET haplotype prevalence in Zone 5**		
Factor	**OR***	**95% CI****	**p-value**	**OR***	**95% CI****	**p-value**	**OR***	**95% CI****	**p-value**
Study year	1.66	0.63-4.37	0.287	7.95	2.24-28.19	0.006	1.18	0.35-3.97	0.771
Age of participant (years)	0.98	0.94-1.02	0.264	0.99	0.82-1.19	0.867	0.98	0.93-1.03	0.322
Residence in a peri-urban area vs. a rural area	1.02	0.52-1.99	0.958	Omitted***			1.10	0.44-2.72	0.830
Gender	1.01	0.69-1.47	0.978	1.03	0.56-2.33	0.928	1.00	0.62-1.61	0.995

*Odds ratio
**95% confidence interval
***excluded from the model because of co-linearity

In Zone 4 the *pfcrt* CVIET haplotype prevalence increased from 18.52% (5/27) in 2010 to 66.67% (42/63) by 2011 not accounting for survey design. In the logistic regression model, this increase was found to be significant (OR: 8.80; 95% CI: 2.50-30.94; *P*=0.005) (Figure 4.2). None of the factors: the age of participants (OR: 0.98; 95% CI: 0.83-1.15; *P*=0.737) or gender (OR: 0.94; 95% CI: 0.44-1.99; *P*=0.848) were found to influence this increase, a finding confirmed by multivariate regression analysis (Table 4.1). Residence in a peri-urban area vs. a rural area was omitted from the model due to co-linearity.

In Zone 5, the *pfcrt* CVIET haplotype prevalence increased from 37.16% (55/148) in 2010 to 40.18% (88/219) by 2011 (Figure 4.2) not accounting for survey design. However, this increase had no statistical significance (OR: 1.14; 95% CI: 0.32-4.08; *P*=0.830) after logistic regression analysis. As in Zone 4, none of the factors: the age of participants (OR: 0.98; 95% CI: 0.93-1.02; *P*=0.300); gender (OR: 1.01; 95% CI: 0.64-1.58; *P*=0.976) or residence in a peri-urban area vs. a rural area (OR: 1.05; 95% CI: 0.42-2.63; *P*=0.917) were associated with *pfcrt* CVIET haplotype prevalence which was also confirmed by multivariate regression analysis (Table 4.1).

The presence of the *pfcrt* CVIET haplotype was not affected by the number of clonal infections across the study area (OR: 1.16.; 95% CI: 0.75-1.80; *P*=0.486); in Zone 4 (OR: 0.68; 95% CI: 0.18-2.65; *P*=0.518) or Zone 5 (OR: 1.45; 95% CI: 0.93-2.29; *P*=0.091).

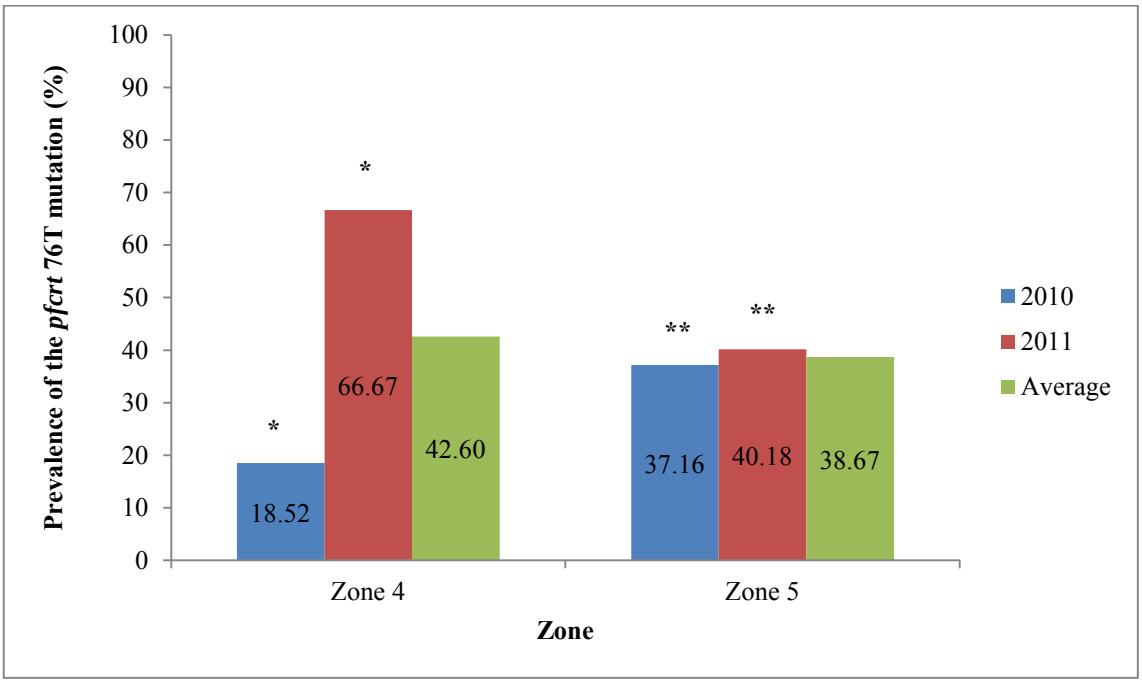


Figure 4.2: Prevalence of the *pfcrt* CVIET haplotype associated with chloroquine resistance in Zone 4 and Zone 5 in 2010 and 2011. The average indicates the change in parasite prevalence over the study period in Zones 4 and 5. **P*=0.005 ** *P*=0.830.

4.2.2. *pfmdr1* copy number and *pfmdr1* N86Y resistance marker prevalence

Copy number amplification data was obtained from 61.78% (485/596) of the PCR-confirmed *falciparum* positive samples. All samples analysed had a single copy of the *pfmdr1* gene.

Codon 86 of the *pfmdr1* gene was successfully amplified in 544 of the 596 (91.28%) PCR-confirmed *P.falciparum* positive samples. An image of amplified fragments containing the *pfmdr1* N86Y codon subjected to AflIII restriction digestion is displayed in Figure 4.3.

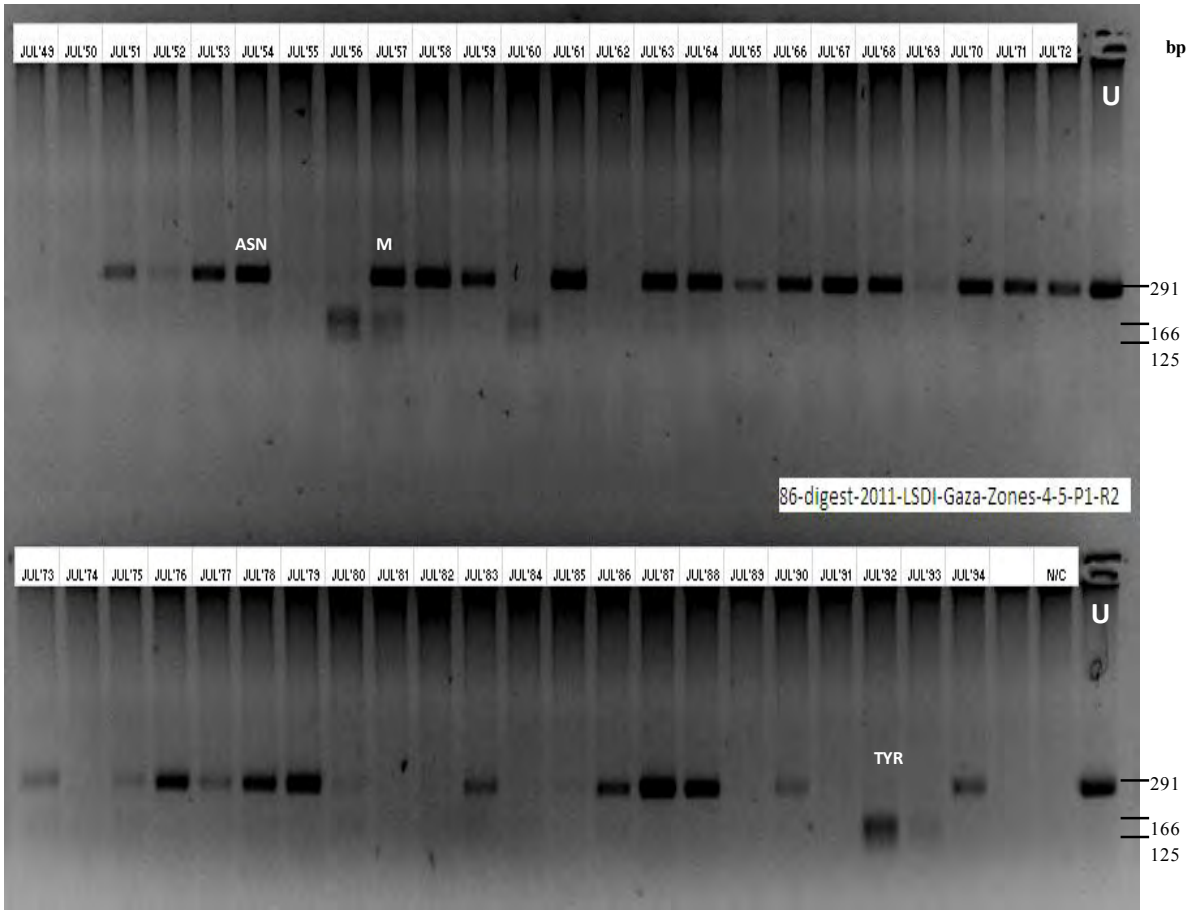


Figure 4.3: An image of a 2% agarose gel showing the restriction fragments obtained following the digestion of amplified products containing the *pfmdr1* 86 allele with the restriction enzyme AflIII. The label above each well is the unique laboratory identity assigned to each sample. An undigested PCR amplified DNA fragment (U) containing the *pfmdr1* 86 allele was used as a molecular marker to differentiate between wildtype and mutant alleles. The presence of a single uncut fragment (291 bp) denotes a sensitive genotype, with the amino acid asparagine (ASN) at codon 86 in the *pfmdr1* gene. The presence of a smaller fragment (166 bp) denotes the presence of a *pfmdr1* mutant allele with the amino acid tyrosine (TYR) replacing asparagine at *pfmdr1* codon 86. M indicates a sample is carrying two or more *P. falciparum* parasite populations, one of which is wildtype at *pfmdr1* 86 and one mutant at *pfmdr1* 86.

The *pfmdr1* 86Y mutation, associated with lumefantrine sensitivity but chloroquine resistance, decreased from 35.32% (77/218) in 2010 to 15.03% (49/326) in 2011 showing statistical significance (OR: 0.32; 95% CI: 0.14-0.77; *P*= 0.014) as confirmed by multivariate regression analysis (Table 4.2). The prevalence of this mutation was not associated with the age of participants (OR: 0.95; 95% CI: 0.89-1.01; *P*=0.078) or gender (OR: 1.17; 95% CI: 0.77-1.78; *P*=0.437). Residence in a peri-urban area appeared to reduce the chances of being infected with a parasite carrying the mutant *pfmdr1* 86Y allele (OR: 0.61; 95% CI: 0.39-0.94; *P*=0.027). This association was confirmed by multivariate regression analysis (Table 4.2).

Table 4.2: Output from a multivariate analysis of predefined factors associated with prevalence of the *pfmdr1* 86Y mutation in Zones 4 and 5, together and as individual zones, between 2010 and 2011

	***pfmdr1* 86Y mutation prevalence**			***pfmdr1* 86Y mutation prevalence in Zone 4**			***pfmdr1* 86Y mutation prevalence in Zone 5**		
Factor	**OR***	**95% CI****	**p-value**	**OR***	**95% CI****	**p-value**	**OR***	**95% CI****	**p-value**
Study year	0.31	0.13-0.71	0.009	0.22	0.08-0.55	0.006	0.34	0.11-1.05	0.059
Age of participant (years)	0.95	0.89-1.01	0.075	0.95	0.79-1.14	0.529	0.94	0.87-1.01	0.073
Residence in a peri-urban area vs. a rural area	0.57	0.37-0.89	0.016	Omitted***			0.65	0.41-1.02	0.061
Gender	1.12	0.75-1.69	0.554	0.72	0.33-1.56	0.344	1.26	0.78-2.04	0.309

*Odds ratio
**95% confidence interval
***excluded from the model because of co-linearity

In Zone 4 the *pfmdr1* 86Y mutation prevalence decreased from 46.94% (23/49) in 2010 to 18.03% (11/61) in 2011 (Figure 4.4) not accounting for survey design. This decrease was statistically significant (OR: 0.25; 95% CI: 0.11-0.58; *P*= 0.006) when computed in a logistic regression model. In Zone 5, a decrease from 31.95% (54/169) in 2010 to 14.34% (38/265) in 2011 was observed (Figure 4.4) before adjusting for survey design. After running a logistic regression model, this decrease held no statistical significance (OR: 0.36; 95% CI: 0.11-1.12; *P*= 0.074). None of the other factors were associated with *pfmdr1* 86Y mutation prevalence in Zone 4: the age of participants (OR: 0.97; 95% CI: 0.82-1.16; *P*=0.721) and gender (OR: 0.90; 95% CI: 0.37-2.20; *P*=0.789) as confirmed by multivariate regression analysis (Table 4.2). Area of residence was omitted from the analysis due to co-linearity. Similarly, there were no associations in Zone 5: the age of participants (OR: 0.93; 95% CI: 0.87-1.00; *P*=0.057); gender (OR: 1.28; 95% CI: 0.77-2.13; *P*=0.308) or residence in a peri-urban area vs. a rural area (OR: 0.71; 95% CI: 0.45-1.10; *P*=0.111) as confirmed by multivariate regression analysis (Table 4.2).

Mixed *pfmdr1* 86 alleles were detected in 14.95% (83/555) of the samples analysed. The association between multiplicity of infection (2 or more infections) and *pfmdr1* 86Y resistance

marker prevalence held no significance (OR: 0.98; 95% CI: 0.68-1.42; *P*=0.918) in the study area as in Zone 4 (OR: 0.73; 95% CI: 0.26-2.04; *P*=0.478) and Zone 5 (OR: 1.10; 95% CI: 0.73-1.64; *P*=0.620).

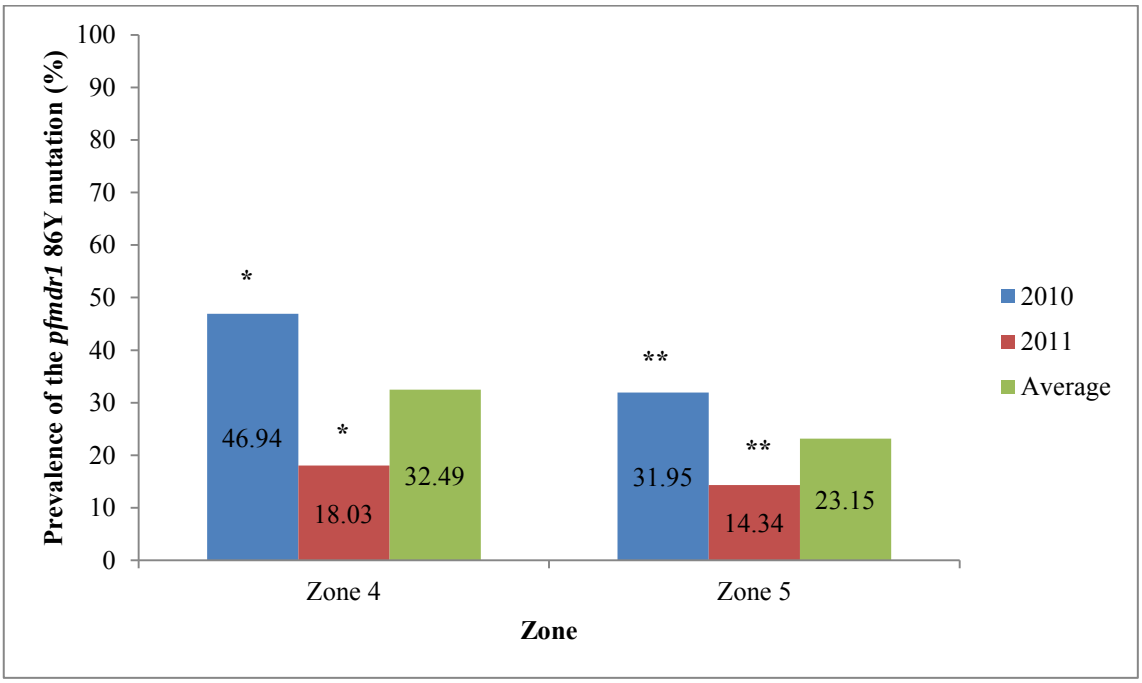


Figure 4.4: Prevalence of the *pfmdr1* 86Y mutation associated with lumefantrine resistance and chloroquine sensitivity in Zones 4 and 5 in 2010 and 2011 The average indicates the change in parasite prevalence over the study period in Zones 4 and 5. **P*=0.006 ** *P*=0.074.

4.2.3. Associations between chloroquine, artemether-lumefantrine and sulphadoxine-pyrimethamine resistance marker prevalence

The associations between the presence of the *pfcrt* CVIET haplotype, *pfmdr1* 86Y and SP quintuple mutations were investigated. There was a positive non-statistical association between the presence of the *pfcrt* CVIET haplotype and the *pfmdr1* 86Y mutations between 2010 and 2011 (OR: 1.37; 95% CI: 0.80-2.34; *P*=0.234). Multivariate logistic regression analysis confirmed this non-statistical negative association after adjusting for the age of participants, gender and residence in a peri-urban area vs. a rural area (Table 4.3). There was a negative association between the *pfcrt* CVIET haplotype and the *pfmdr1* 86Y mutation prevalence in Zone 4 (OR: 0.40; 95% CI: 0.20-0.80; *P*=0.016) which was confirmed by multivariate logistic regression (Table 4.3). In Zone 5, the *pfcrt* CVIET haplotype prevalence was positively associated with the *pfmdr1* 86Y mutation prevalence (OR: 1.85; 95% CI: 1.01-3.40; *P*=0.047) as confirmed by multivariate logistic regression (Table 4.3).

There was no association between the *pfcrt* CVIET haplotype prevalence and SP quintuple mutation prevalence in the study area between 2010 and 2011(OR: 0.62; 95% CI: 0.30-1.25; *P*=0.170) as was observed in Zone 4 (OR: 0.74; 95% CI: 0.28-1.95; *P*=0.482) and Zone 5 (OR: 0.73; 95% CI: 0.37-1.42; *P*=0.316) and confirmed by multivariate logistic regression after adjusting for the age of participants, gender and residence (Table 4.3).

The association of the *pfmdr1* 86Y mutation prevalence with SP quintuple mutation prevalence was also computed. The presence of the *pfmdr1* 86Y mutation was negatively associated with SP resistance marker prevalence over the study period after adjusting for the age of participants, gender and residence (OR: 0.34; 95% CI: 0.16-0.71; *P*=0.006). While no significant association between the *pfmdr1* 86Y mutation prevalence and sulphadoxine-pyrimethamine resistance marker prevalence was observed in Zone 4 (OR: 0.76; 95% CI: 0.25-2.31; *P*=0.579); a significant negative association was observed in Zone 5 (OR: 0.22; 95% CI: 0.08-0.54; *P*=0.004). Residence in a peri urban area vs. a rural area was shown to influence the association between the *pfmdr1* 86Y mutation and sulphadoxine-pyrimethamine (SP) resistance marker prevalence (Table 4.4).

Table 4.3: Output from a multivariate analysis of predefined factors associated with prevalence of the *pfcrt* CVIET haplotype in Zones 4 and 5 between 2010 and 2011 in the presence of the *pfmdr1* 86Y mutation and the sulphadoxine-pyrimethamine (SP) quintuple mutation prevalence

	***pfcrt* CVIET haplotype prevalence**			***pfcrt* CVIET haplotype prevalence in Zone 4**			***pfcrt* CVIET haplotype prevalence in Zone 5**		
Factor	**OR***	**95% CI****	**p-value**	**OR***	**95% CI****	**p-value**	**OR***	**95% CI****	**p-value**
Study year	1.18	0.70-4.68	0.206	9.20	2.6-41.06	0.010	1.29	0.40-4.22	0.642
Age of participant (years)	0.97	0.93-1.02	0.239	0.94	0.76-1.17	0.531	0.98	0.94-1.03	0.439
Residence in a peri-urban area vs. a rural area	0.99	0.5-1.93	0.967	Omitted***			1.06	0.44-2.57	0.885
Gender	0.98	0.63-1.53	0.928	0.95	0.36-2.54	0.911	1.01	0.59-1.71	0.972
SP quintuple mutation	0.67	0.33-1.34	0.244	0.66	0.12-3.58	0.578	0.88	0.43-1.84	0.719
pfmdr1 86Y mutation	1.47	0.89-2.41	0.122	0.61	0.19-1.92	0.344	1.86	1.04-3.33	0.040

*Odds ratio
**95% confidence interval
***excluded from the model because of co-linearity

Table 4.4: Output from a multivariate analysis of predefined factors associated with prevalence of the *pfmdr1* 86Y mutation in Zones 4 and 5 between 2010 and 2011 in the presence of sulphadoxine-pyrimethamine (SP) resistance

	***pfmdr1* 86Y mutation prevalence**			***pfmdr1* 86Y mutation prevalence in Zone 4**			***pfmdr1* 86Y mutation prevalence in Zone 5**		
Factor	**OR***	**95% CI****	**p-value**	**OR***	**95% CI****	**p-value**	**OR***	**95% CI****	**p-value**
Study year	0.31	0.13-0.71	0.008	0.41	0.15-1.13	0.075	0.32	0.11-1.00	0.043
Age of participant (years)	0.96	0.89-1.03	0.230	0.98	0.82-1.17	0.783	0.95	0.87-1.04	0.253
Residence in a peri-urban area vs. a rural area	0.54	0.32-0.91	0.024	Omitted*			0.54	0.31-0.95	0.034
Gender	1.04	0.71-1.53	0.821	0.76	0.28-2.06	0.522	1.16	0.74-1.79	0.486
SP resistance prevalence	0.35	0.14-0.83	0.020	0.62	0.15-2.56	0.440	0.19	0.07-0.57	0.007

*Odds ratio
**95% confidence interval
***excluded from the model because of co-linearity

4.3. Discussion

4.3.1. Prevalence of the *pfcrt* chloroquine resistance marker in Zones 4 and 5

This study reports on the prevalence of chloroquine resistance markers nine years after this drug was removed as first-line treatment in Gaza Province. The chloroquine resistance marker prevalence is 46.10% in the study area over the study period with an eight-fold increase observed in Zone 4 between 2010 and 2011. This contrasts a research study in the area which reported a steady decline of chloroquine resistance marker prevalence in the study area where it decreased from 96.1% in 2006 to 32.36% in 2010 (Raman *et al*., 2011). As resistance marker prevalence is sometimes an indicator of drug use and resistant parasites have a fitness advantage when drug pressure is sustained, (Frosch *et al*., 2011; Laufer and Plowe, 2004), the increase in chloroquine

resistance marker prevalence observed in this study suggests that chloroquine is still available and used in Gaza Province.

Although chloroquine was removed from the national drug treatment policy nine years ago, this removal may not have been enforced. Wildtype *P. falciparum* strains have previously been observed to re-emerge following the withdrawal of chloroquine in Africa (Frosch *et al*., 2011; Kublin *et al*., 2003). In Kenya and Tanzania this rate of decline was slow, whilst chloroquine sensitive strains re-emerged relatively quicker ten years after chloroquine was removed from circulation in Malawi. In Malawi, the *pfcrt* 76T marker decreased in prevalence from 85% in 1992 to 0% by 2001 (Frank *et al*., 2011; Kublin *et al*., 2003). This significant downward trend has been attributed, in part, to the strict removal of chloroquine in the public and private sectors in Malawi (Frosch *et al*., 2011; Laufer *et al*., 2006). As such strict controls were not enforced in Gaza Province, low level chloroquine usage may contribute to the slow decline in chloroquine resistance markers.

Despite the national drug policy change replacing the use of chloroquine for treatment of uncomplicated malaria in Gaza Province in 2002, it is possible that chloroquine is still used as self treatment of the disease. Self-treatment is not uncommon in Africa, especially in areas where drugs are readily available in local shops. This easy access to drugs leads to unnecessary drug use and increased drug pressure (Hopkins *et al*., 2007; Hume *et al*., 2008; Wafula *et al*., 2012). Whilst sentinel sites in Zones 4 and 5 were selected for the survey because of its close proximity to a health care facility and national roads, it is possible that self-treatment could have occurred in this region probably influenced by distance from and travelling costs to the health facility, household socio-economic status, household heads' age and sex, parents' education and unavailability of ACTs (Beiersmann *et al*., 2007; Hopkins *et al*., 2007).

The contrasting reports of chloroquine resistance marker prevalence in the same zones in Gaza Province by Raman *et al.* (2011) and this study highlights the need for continued monitoring of drug resistance markers in the province so that the possibility of a re-introduction of chloroquine as antimalarial treatment can be considered. The re-introduction of drugs that had previously fallen to resistance such as chloroquine could lighten the financial burden of malaria control efforts, seeing as it is a safer, more cost effective option compared to the expensive treatments currently available and considering the time taken to develop new affordable drugs (Laufer and Plowe, 2004).

4.3.2. Prevalence of the *pfmdr1* markers of lumefantrine and chloroquine resistance in Zones 4 and 5

In this study, copy number amplification of the *pfmdr1* gene was not observed. Copy number amplification of the *pfmdr1* gene is driven by drug pressure (Anderson *et al*., 2009) and copy number changes occur at a higher rate than point mutations although selective events differ from those associated with point mutations (Nair *et al*., 2007). Copy number amplification may confer resistance to both drugs in a combination therapy (Price *et al*., 2006) and is a worrying indicator of resistance. Any selection pressure associated with artemether-lumefantrine is due to lumefantrine as the ACT selects for resistance to the partner drug (Alker *et al*., 2007). Since artemether-lumefantrine use in Gaza Province was only implemented in early 2010, drug pressure may not be high, which may explain why only single copies of the *pfmdr1* gene have been observed. Copy number amplification of the *pfmdr1* gene may also not have been observed in this study since large chunks of chromosome 5 rather than the *pfmdr1* gene alone is usually amplified which may be disadvantageous and could impose fitness costs (Anderson *et al*., 2009).

As previous studies suggest, the *pfmdr1* 86N polymorphism is associated with increased sensitivity to chloroquine and reduced *in vitro* susceptibility to lumefantrine (Andriantsoanirina *et al*., 2010; Dokomajilar *et al*., 2006; Humphreys *et al*., 2007; Mwai *et al*., 2012). In this study, a decrease in the *pfmdr1* 86Y mutation which is thought to modulate chloroquine resistance was observed and may be an indicator of decreasing chloroquine resistance in Gaza Province. However, the increase in prevalence of this marker is a cause for concern regarding continued lumefantrine efficacy. Studies in various African countries have shown that artemether-lumefantrine treatment selects for *P. falciparum* parasites carrying the *pfmdr1* 86N allele (Humphreys *et al*., 2007; Some *et al*., 2010). It is important to continuously monitor this allele and others associated with resistance to artemether-lumefantrine to ensure that the development and spread of resistance to this drug combination is controlled.

4.3.3. Associations between the prevalence of chloroquine, sulphadoxine-pyrimethamine and lumefantrine resistance markers in Zones 4 and 5

The presence of the *pfcrt* CVIET haplotype was negatively associated with the *pfmdr1* 86Y mutation prevalence in Zone 4 but a positive association was observed in Zone 5. Various studies suggest that the association between polymorphisms in the *pfmdr1* gene and chloroquine resistance is thought to be contributed by the genetic background of the parasite strain (Chaijaroenkul *et al*., 2011; Duraisingh and Cowman, 2005; Sa *et al*., 2009; Sanchez *et al*., 2010; Valderramos *et al*., 2010). In this study, it was found that Zone 5 has a higher prevalence of clonal infections than Zone

4 (Section 3.2.3) possibly due to different drug pressures in these two regions, as discussed earlier, which may have contributed to the positive association between *pfcrt* CVIET haplotype prevalence and *pfmdr1* 86Y mutation prevalence.

A negative association between the *pfmdr1* 86Y mutation and SP resistance mutation prevalence was observed in this study. This observation may have a genetic explanation. Resistance to sulphadoxine-pyrimethamine is conferred by mutations that occur in genes encoding drugs targets while the mutations that confer chloroquine resistance occur in drug transporters genes which may be seen as less essential and have lower costs associated with mutations (Hastings and Donelly, 2005). Therefore the *dhfr* triple and *dhps* double mutations that confer sulphadoxine-pyrimethamine drug resistance may have more compensatory mutations to sustain resistance or a lower survival disadvantage than chloroquine resistance conferring mutations (Laufer and Plowe, 2004). As discussed by Griffing *et al.* (2010), this could also be due to inbreeding or the establishment of resistance from populations already fixed for the *pfmdr1* gene suggesting a lack of allelic diversity in the parasite population in Gaza Province. This requires further research into the genetic diversity and allelic background of parasites in Gaza Province, which is beyond the scope of this study. It would be of interest to determine if this association is due to a fitness cost in the *pfmdr1* gene in the presence of SP resistance mutations or if there is a genetic explanation for this association as suggested by Figueiredo *et al*. (2008) when they observed a similar phenomenon.

4.3.4. Conclusion

In conclusion, while this study failed to report a significant increase in chloroquine resistance markers overall, the more than eight-fold increase observed in Zone 4 raises concern over the availability and use of ineffective antimalarial drugs in the province. Studies into drug self-treatment practices and drug availability in the province would prove beneficial to future drug policy implementations and malaria control efforts. Further monitoring of chloroquine resistance markers is required given the contrasting reports of chloroquine resistance in the province to determine if chloroquine sensitivity could return in Gaza Province as observed in other African countries (Frosch *et al*., 2011; Mwai *et al*., 2012). Whilst *pfmdr1* copy number amplification revealed only single copies of the gene in the study population, the high prevalence of the *pfmdr1* 86N polymorphism reported in this study as an indicator of resistance to the partner drug lumefantrine, casts doubt on the use of artemether-lumefantrine use in the region. Given that ineffective partner drugs aid the development and spread of drug resistance, continued monitoring of molecular markers associated with artemisinin resistance is required to ensure that artemether-lumefantrine remains an efficacious treatment for uncomplicated *P. falciparum* malaria in Gaza Province. Antimalarial drug resistance alleles have been shown to spread over large geographical

regions (Laufer and Plowe, 2004) and with the increased use of artemether-lumefantrine in sub-Saharan Africa (Baliraine and Rosenthal, 2011), the monitoring of molecular markers associated with drug resistance in regions surrounding Gaza Province should also be considered.

CHAPTER 5: Conclusion and future perspectives

Malaria, a parasitic disease, remains a public health, economic and social burden in many malaria endemic countries. While a variety of factors including insecticide resistance, drug counterfeiting, poor adherence to control practices and high costs of treatment have contributed to the sustained burden of malaria, one of the most important factors is antimalarial drug resistance. Whilst clinical trials are the gold standard in drug efficacy monitoring, they are costly, labour intensive and time consuming. In areas of low transmission there is added concern of recruiting the required number of participants. As molecular markers associated with treatment failure have been identified for most antimalarials, the routine surveillance for these molecular markers is a feasible alternative to clinical trials. Picot *et al*. (2009) argued that data from molecular studies can be used to inform treatment policy. This study reports on the prevalence of molecular markers associated with sulphadoxine-pyrimethamine (SP), chloroquine and artemether-lumefantrine resistance in two predefined municipal zones in Gaza Province two years after artemether-lumefantrine was rolled out as first-line treatment for uncomplicated malaria at all health facilities in the province.

Despite the continuous implementation of integrated malaria control measures during the study period (Lubombo Spatial Development Initiative, 2009), asexual parasite prevalence was similar to that reported by a similar study in the area (Raman *et al*., 2011). This observation does however show that the previous malaria control interventions conducted in the region such as vector control using indoor residual spraying, prompt diagnosis using rapid diagnostic tests and effective treatment of malaria cases using artemisinin-based combination therapies (Lubombo Spatial Development Initiative, 2009) have been effective in preventing further morbidity and mortality associated with the disease.

Sulphadoxine-pyrimethamine either as a monotherapy or in combination with a partner drug had been removed as a drug option and is thought to not have been used for the treatment of uncomplicated malaria in Gaza Province since the 2008/2009 malaria season, however, resistance markers associated with sulphadoxine-pyrimethamine resistance and treatment failure were approaching saturation in the province by 2011. This high SP resistance marker prevalence supports the drug policy change from artesunate plus sulphadoxine-pyrimethamine to artemether-lumefantrine adopted by the Mozambican government in 2008. The level of SP resistance marker prevalence observed in this study does however cast doubt on the use of sulphadoxine-pyrimethamine for intermittent preventive treatment for pregnant women in the region as resistance is thought to hinder the drugs' efficacy (Harrington *et al*., 2011). This conflicts with some reports that suggest that sulphadoxine-pyrimethamine resistance does not affect the drugs' use as a

preventive therapy (ter Kuile *et al*., 2005). Further study is therefore required to determine the effect of resistance on the preventive ability of drugs used for intermittent preventive treatment.

Of some concern was the increase, particularly in Zone 4, of the chloroquine resistance marker. This result differs from a similar study which showed that chloroquine resistance markers were decreasing markedly in the same sites in the region (Raman *et al*., 2011). Possible reasons for this increase include changes in treatment seeking behaviour, population movement and community and structural development generally observed in rural areas compared to urban areas (Keiser *et al*., 2004) and possible unavailability of ACTs. While chloroquine was discontinued as a first-line antimalarial treatment in 2002 in malaria treatment policies, it is unclear whether this was truly enforced at the community level in Gaza Province. Chloroquine could therefore still be widely available for treatment of malaria in the province. Therefore, an investigation of the antimalarial drugs available at shops, pharmacies and health facilities within the area would prove beneficial to malaria control programmes in the region.

Interestingly the *pfmdr1* 86Y mutation, which modulates chloroquine resistance, decreased over the study period. This may be an indication of a reduction in the degree of chloroquine resistance exhibited by the parasite population in Gaza Province. While the decrease in the *pfmdr1* 86Y allele is positive news regarding a potential re-emergence of chloroquine sensitive parasites, it also acts as a warning of possible reduced lumefantrine efficacy. Previous studies have shown that lumefantrine rapidly selects for the *pfmdr1* 86N allele (Happi *et al*., 2009; Mwai *et al*., 2012). Due to the sustained decline in the *pfmdr1* 86Y genotype, the continued monitoring of this allele is recommended. Studies in Asia showed that copy number amplification of the *pfmdr1* gene is associated with lumefantrine resistance (Price *et al*., 2004; Sisowath *et al*., 2005). No amplification has been previously reported in Africa (Anderson and Roper, 2005), with this study corroborating this finding.

The *pfmdr1* 86Y allele was found to be associated with both the chloroquine resistance marker and the SP quintuple mutation. Residence in a peri-urban vs. a rural area seemed to influence both these associations possibly due to different drug pressures in each area.

In this study, associations between drug resistance and gender, residence in a peri-urban area, the age of participants and febrility were assessed. Of these, only gender and residence in a peri-urban area were found to be associated with drug resistance. As febrility was recorded in only 1.4% of PCR-confirmed RDT positive samples, it was excluded from the analysis. While febrility is a symptom of malaria, the absence of a fever does not always rule out malaria infection (Guinovart *et al*., 2008). The absence of febrility is seen as an indicator of immunity to malaria possibly due to

non-exposure to infection or a protective effect conferred by blood stage immunity (Bejon *et al*., 2009). While the effect of malaria infection on body temperature varies between individuals, the incongruence between the percentage of febrile participants and the percentage of infected participants observed in this study attests to the presence of asymptomatic malaria infections and the development of premunition in malaria endemic areas (Mabunda *et al*., 2009).

The age of participants was not found to be associated with the molecular markers of drug resistance in this study. Given that children at younger ages often present with symptomatic malaria in regions of endemicity (Idro *et al*., 2006), the lack of age association in this study was unexpected. A sampling bias was however discovered which may have skewed the data in some sentinel sites where only six to ten year olds were surveyed. This bias highlights the importance of survey design and preparation in cross-sectional studies where, as observed in this study, extreme weather conditions influenced the sample collection process.

In conclusion, the results from this study contribute to the body of knowledge pertaining to antimalarial drug resistance marker prevalence in Gaza Province. The nearing fixation of the SP resistance markers is a cause of concern regarding the continued efficacy of intermittent preventive treatment using sulphadoxine-pyrimethamine. he success of the artemether-lumefantrine treatment failure has already been reported in East and West Africa, combination lies in the parasites tolerability to the partner drug (Happi *et al*., 2009). Lumefantrine drives the selection for *pfmdr1* 86N allele in malaria endemic areas (Dokomajilar *et al*., 2006; Hastings and Ward, 2005; Humphreys *et al*., 2007; Sisowath *et al*., 2009) therefore the increase in the *pfmdr1* 86N marker serves as an early warning of the development of resistance to the artemether-lumefantrine combination. As artemisinin-based combination therapies for first-line treatment of *P. falciparum* malaria is adopted by over 40 countries in sub-Saharan Africa and artemether-lumefantrine is the ACT of choice recommended by the WHO (Barnes *et al*., 2009; World Health Organization, 2009b), this study also highlights the need for continued monitoring of molecular markers of drug resistance. It also testifies to the importance of molecular surveillance studies in integrated malaria control efforts given the ability of drug resistance to spread across borders (O'Meara *et al*., 2010; Tatem and Smith, 2010; World Health Organization, 2011a).

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APPENDIX

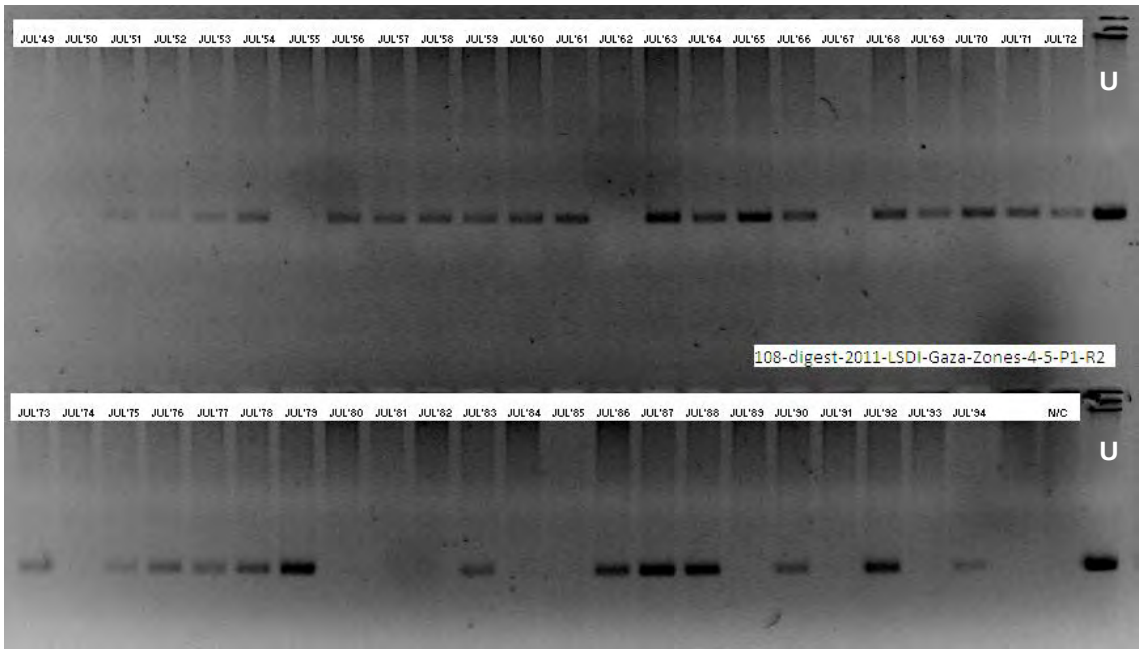

Figure A1: An image of a 2% agarose gel showing the restriction fragments obtained following the digestion of amplified products containing the *dhfr* 108 allele with the restriction enzyme AluI. The label above each well is the unique laboratory identity assigned to each sample. An undigested PCR amplified DNA fragment containing the *dhfr* 108 allele was used as a molecular marker to differentiate between wildtype and mutant alleles. The presence of a single uncut fragment (254 bp) denotes a resistant genotype, with the amino acid asparagine at codon 108 in the *dhfr* gene. The presence of a smaller fragment (210 bp) denotes the presence of a *dhfr* sensitive allele with the amino acid serine present at *dhfr* codon 108 (not observed in this picture).

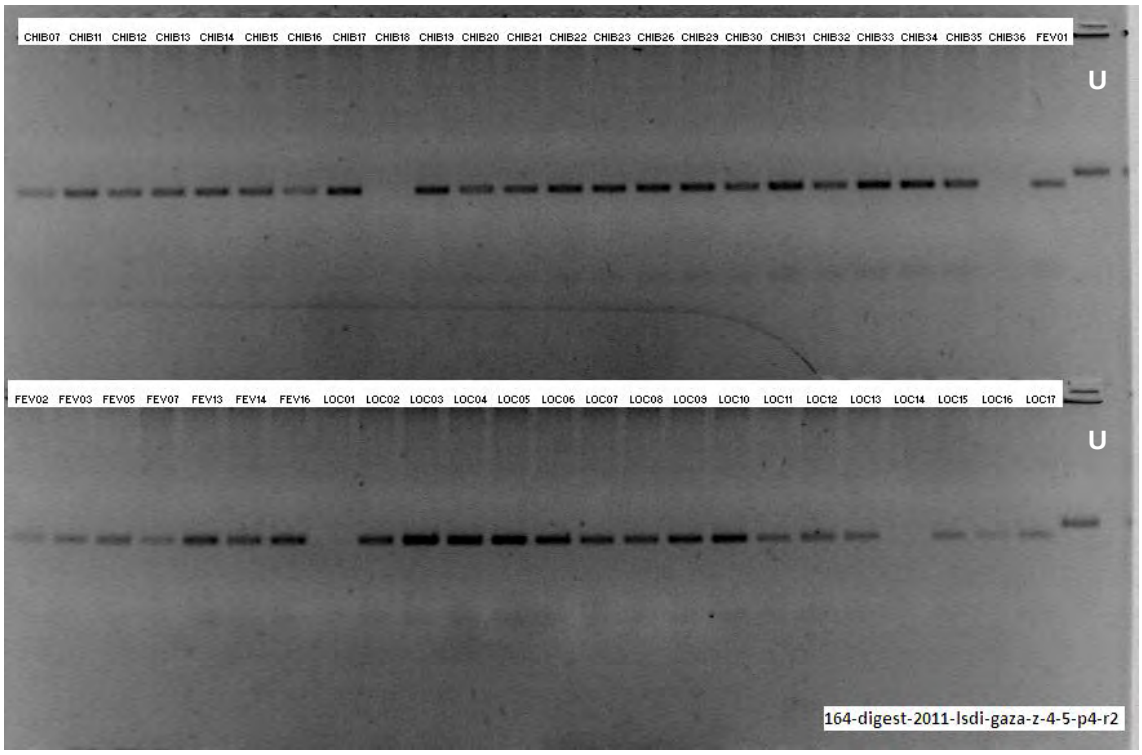

Figure A2: An image of a 2% agarose gel showing the restriction fragments obtained following the digestion of amplified products containing the *dhfr* 164 allele with the restriction enzyme PsiI. The label above each well is the unique laboratory identity assigned to each sample. An undigested PCR amplified DNA fragment containing the *dhfr* 164 allele was used as a molecular marker to differentiate between wildtype and mutant alleles. The presence of a single uncut fragment (254 bp) denotes a resistant genotype, with the amino acid leucine at codon 164 in the *dhfr* gene (not observed in this study). The presence of a smaller fragment (214 bp) denotes the presence of a *dhfr* sensitive allele with the amino acid isoleucine *dhfr* codon 164.

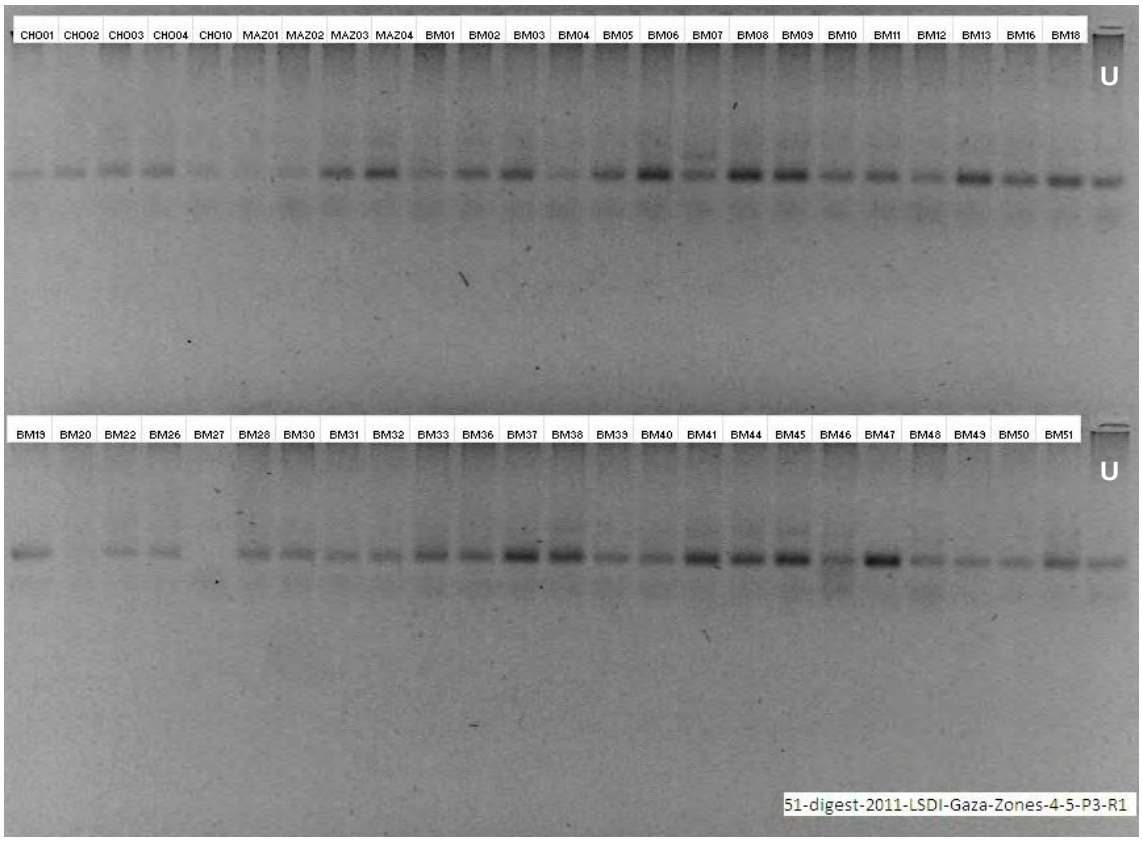

Figure A3: An image of a 2% agarose gel showing the restriction fragments obtained following the digestion of amplified products containing the *dhfr* 51 allele with the restriction enzyme EcoRI. The label above each well is the unique laboratory identity assigned to each sample. An undigested PCR amplified DNA fragment containing the *dhfr* 51 allele was used as a molecular marker to differentiate between wildtype and mutant alleles. The presence of a single uncut fragment (113 bp) denotes a resistant genotype, with the amino acid isoleucine at codon 51 in the *dhfr* gene. The presence of a smaller fragment (78 bp) denotes the presence of a *dhfr* sensitive allele with the amino acid asparagine present at *dhfr* codon 51 (not observed in this image).

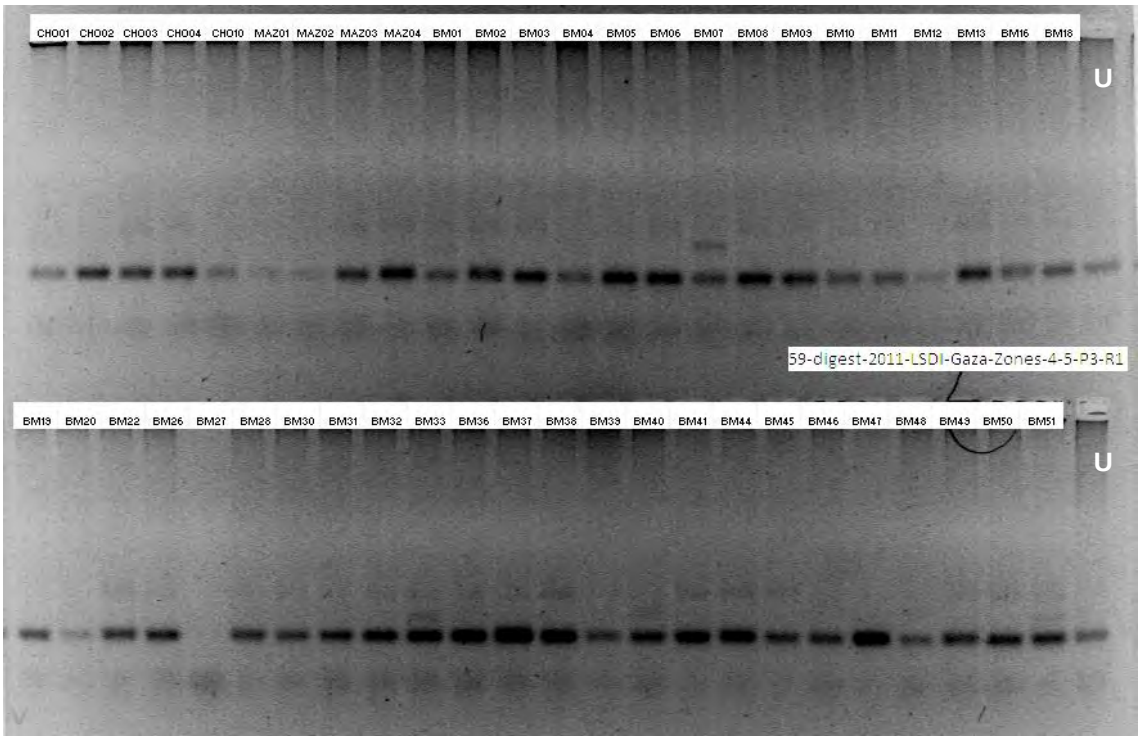

Figure A4: An image of a 2% agarose gel showing the restriction fragments obtained following the digestion of amplified products containing the *dhfr* 59 allele with the restriction enzyme BsrGI. The label above each well is the unique laboratory identity assigned to each sample. An undigested PCR amplified DNA fragment containing the *dhfr* 59 allele was used as a molecular marker to differentiate between wildtype and mutant alleles. The presence of a single uncut fragment (113 bp) denotes a resistant genotype, with the amino acid arginine at codon 59 in the *dhfr* gene. The presence of a smaller fragment (65 bp) denotes the presence of a *dhfr* sensitive allele with the amino acid cysteine present at *dhfr* codon 59 (not observed in this image).

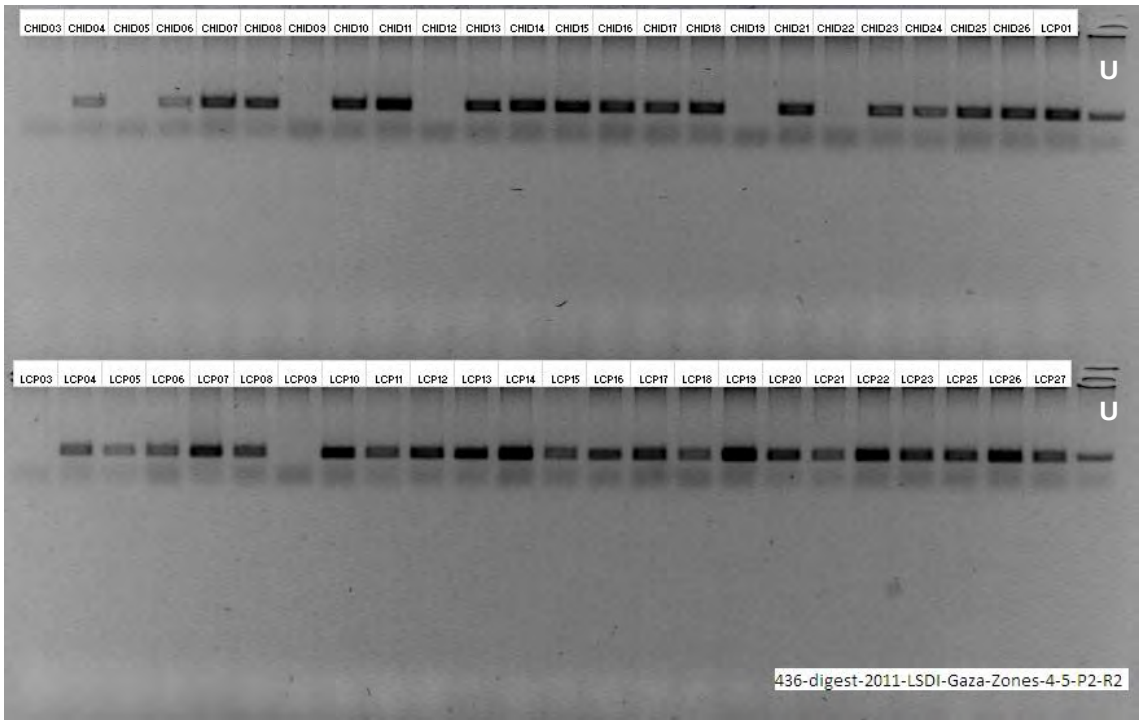

Figure A5: An image of a 2% agarose gel showing the restriction fragments obtained following the digestion of amplified products containing the *dhps* 436 allele with the restriction enzyme MspA1I. The label above each well is the unique laboratory identity assigned to each sample. An undigested PCR amplified DNA fragment containing the *dhps* 436 allele was used as a molecular marker to differentiate between wildtype and mutant alleles. The presence of a single uncut fragment (148 bp) denotes a sensitive genotype, with the amino acid serine at codon 436 in the *dhps* gene. The presence of a smaller fragment (79 bp) denotes the presence of a *dhps* mutant allele with the amino acid alanine replacing serine at *dhps* codon 436 (not observed in this study).

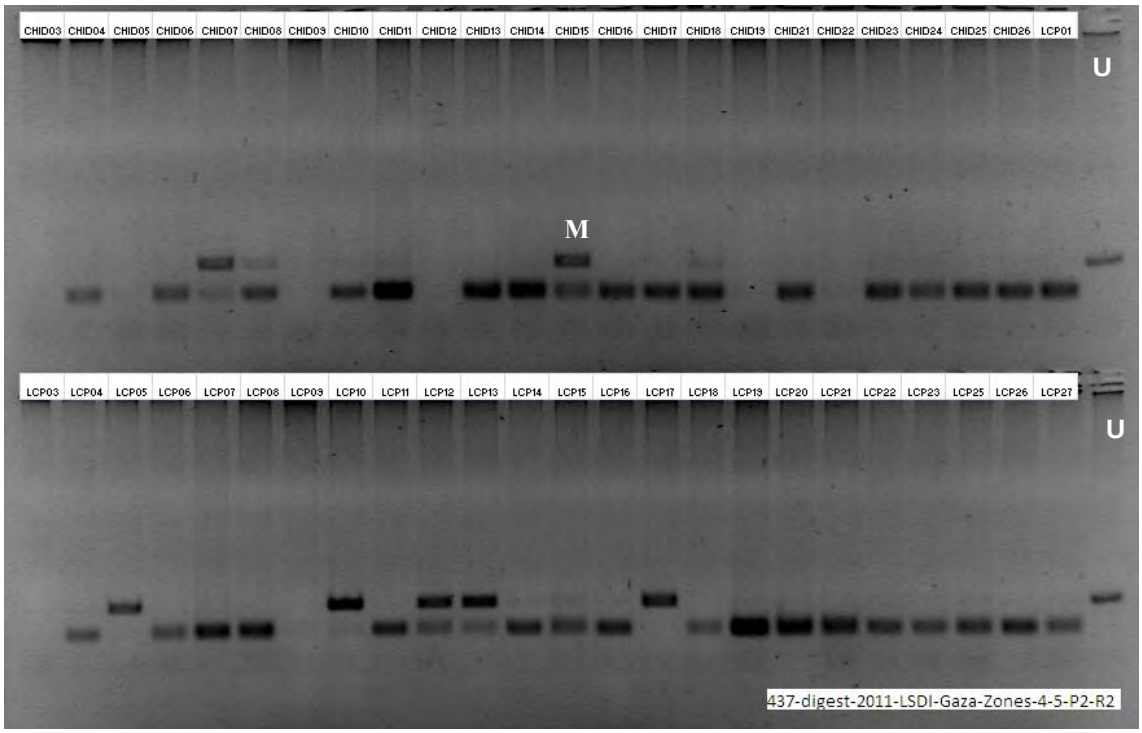

Figure A6: An image of a 2% agarose gel showing the restriction fragments obtained following the digestion of amplified products containing the *dhps* 437 allele with the restriction enzyme AvaII. The label above each well is the unique laboratory identity assigned to each sample. An undigested PCR amplified DNA fragment containing the *dhps* 437 allele was used as a molecular marker to differentiate between wildtype and mutant alleles. The presence of a single uncut fragment (148 bp) denotes a sensitive genotype, with the amino acid alanine at codon 437 in the *dhps* gene. The presence of a smaller fragment (79 bp) denotes the presence of a *dhps* mutant allele with the amino acid glycine replacing alanine at *dhps* codon 437. M indicates a sample is carrying two or more *P. falciparum* parasite populations, one of which is wildtype at *dhps* 437and one mutant at *dhps* 437.

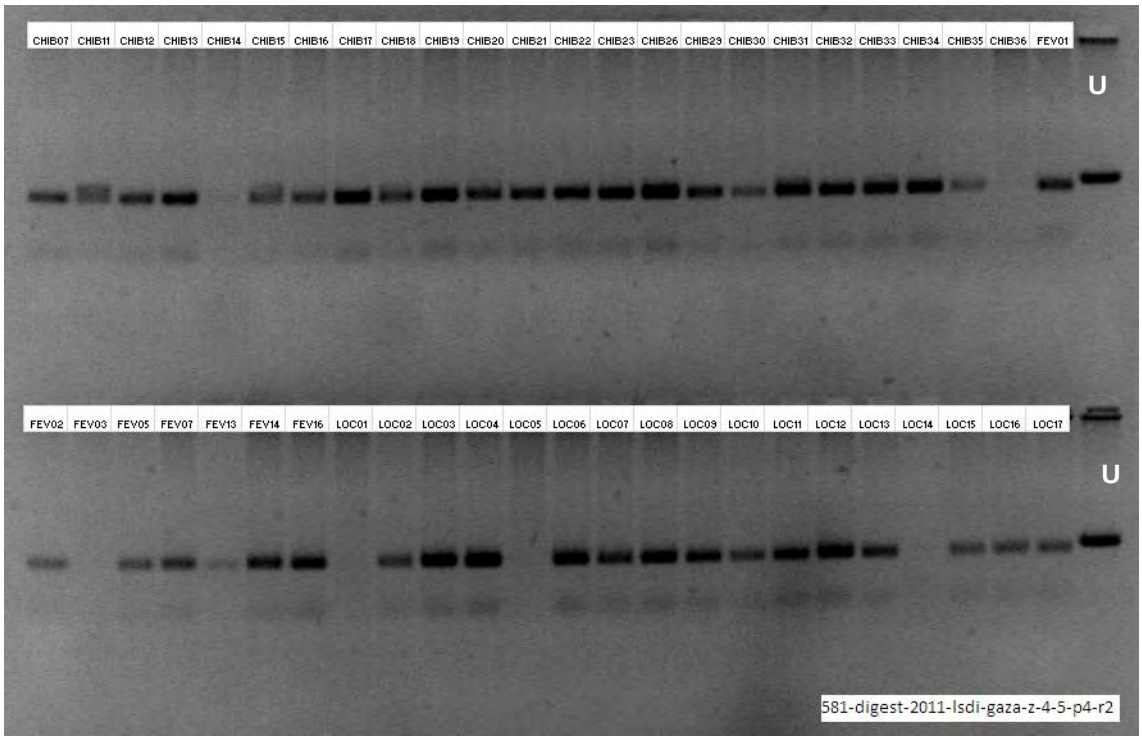

Figure A7: An image of a 2% agarose gel showing the restriction fragments obtained following the digestion of amplified products containing the *dhps* 581 allele with the restriction enzyme MwoI. The label above each well is the unique laboratory identity assigned to each sample. An undigested PCR amplified DNA fragment containing the *dhps* 581 allele was used as a molecular marker to differentiate between wildtype and mutant alleles. The presence of a single uncut fragment (201 bp) denotes a resistant genotype, with the amino acid glycine at codon 581 in the *dhps* gene (not observed in this study). The presence of a smaller fragment (154 bp) denotes the presence of a *dhps* sensitive allele with the amino acid alanine present at *dhps* codon 581.