DETERMINATION OF *PLASMODIUM FALCIPARUM* PARASITE DIVERSITY USING MSPII FAMILY SPECIFIC ALLELIC PRIMERS AMONG SYMPTOMATIC CHILDREN AGED FIVE YEARS AND BELOW IN ZAMBIA

by

MOONGA. B. HAWELA

A dissertation submitted in partial fulfillment of the

requirements for the degree of Master of Science in Medical

Parasitology
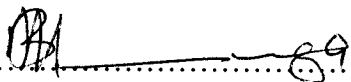
The University of Zambia

December 2008

DECLARATION

This dissertation is the original work of **MOONGA BELDEN HAWELA**

It has been conducted in accordance with the guidelines for MSC. In Medical Parasitology dissertations of the University of Zambia. This dissertation has not been submitted elsewhere for a degree at this or another University.
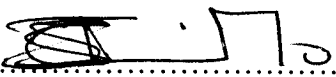
Signature...

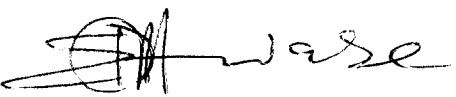Date...........23/03/09...

APPROVAL

The University of Zambia approves this dissertation of Moonga Belden Hawela in partial fulfillment for the requirements for the award of the degree in Master of Medical Parasitology.

Examiner's signature   Date

....   25/3/09
....

....   26/03/09
....

....

ABSTRACT

Introduction: Malaria is still a major public health problem in many tropical and subtropical countries. Therefore effective integrated malaria control strategies are a requirement. However, parasite antigen diversity might be a hurdle in the efficacy of some malaria control strategies like the malaria vaccine against *Plasmodium falciparum* infection if a full repertoire of variant forms is not incorporated into the vaccine. In addition, malaria rapid diagnostic tests (RDTs) offer great potential for rapid and accurate diagnosis of malaria infections. Parasite genetic diversity of target antigens particularly for PfHRP2-based RDTs may affect their sensitivity among other factors.

Methods: In 2006, a prospective analytical study was conducted to determine the diversity of *Plasmodium falciparum* merozoite surface protein 2 (MSP-2) in four sites in Zambia. A nested polymerase chain reaction (PCR) amplification was carried out on two hundred and eighty five dried blood spots with *Plasmodium falciparum* mono infection using primers for the conserved regions of MSP-2 as well as family specific alleles for the variable regions. PCR-restriction fragment length polymorphism analysis of MSP-2 using a HinfI digests of each nested PCR product followed by product detection on a 2.0% agarose gel electrophoresis with ethidium bromide was performed to determine the allelic families and genotypes.

Results: The study findings show that, there are forty six different genotypes of *Plasmodium falciparum* commonly found in Zambia, twenty four allelic genotypes belong to the FC27 allelic family and twenty two from the 3D7 allelic family. The study also indicated that *Plasmodium falciparum* infections carry more than one allelic genotype at each particular time and one infection carried eight different genotypes.

Discussion: These findings show that *Plasmodium falciparum* in Zambia exists in many different allelic genotypes and several patients infected with *Plasmodium falciparum* carry atlest more than one genotype.

Conclusions: This means that the inclusion of all the available genotypes in both the Malaria vaccine and RDTs would be of maximum benefit to the Zambian populations. The multiple *Plasmodium falciparum* infections will have other implications such as having one genotype sensitive the drug and another resistant therefore this needs to be further explored.

ACKNOWLEDGEMENTS

LIST OF ABBREVIATIONS

ACTs	Artemisinin-based Combination Therapy
CBoH	Central Board of Health
CQ	Chloroquine
DBS	Dried blood spots
DHMT	District Health Management Team
IEC	Information, Education and Communication
MACEPA	Malaria Control and Evaluation Partnership In Africa
MIS	Malaria Information Systems
MoH	Ministry of Health
MSP-2	Merozoite surface protein II
NMCC	National Malaria Control Centre
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
RBM	Roll Back Malaria
RDTs	Rapid Diagnostic Tests
RFLP	Restriction fragment length polymorphism genotyping
SP	Sulphadoxine-Pyrimethamine
Spp	Species
UNICEF	United Nations International Children Emergency Fund
UNZA	University of Zambia
WHO	World Health Organisation

Table of Contents   **Page**

Chapter: 1. Introduction and Literature review

Chapter: 2. Materials and methods

DEFINITIONS

Alleles: Alternative forms of a genetic characteristic.

Base pair: Two of the building blocks of DNA held together by weak bonds. In a DNA molecule, adenine always bonds with thymine (A-T), and cytosine always bond s with guanine (C-G).

Clone: A group of genetically identical cells or organisms that are descended from one parent.

Electrophoresis: A method of separating large molecules-such as DNA fragments or proteins-from a mixture of similar molecules.

Gel: A dense network of fine particles dispersed with water. Used to separate different-sized strands of DNA.

Genotype: The entire genetic identity of an individual, including alleles, or gene forms, that do not show as outward characteristics.

Molecular weight size marker: a piece of DNA of known size, or a mixture of pieces with known size, used on electrophoresis gels to determine the size of unknown DNA's by comparison.

Polymerase chain reaction (PCR): A "biological copy machine": a method for making many copies of a specific DNA base sequence.

Restriction enzyme, endonuclease: A protein that recognizes specific, short sequences of DNA and cuts at those sites.

Restriction: To "restrict" DNA means to cut it with a restriction enzyme.

Restriction fragment: The piece of DNA released after restriction digestion of plasmids or genomic DNA.

Sequence: As a noun, the sequence of a DNA is a buzz word for the structure of a DNA molecule, in terms of the sequence of bases it contains.

Taq polymerase: A DNA polymerase isolated from the bacterium Thermophilis aquaticus and which is very stable to high temperatures.

CHAPTER: 1.0 INTRODUCTION AND LITERITURE REVIEW

1.1. Introduction

Malaria is the largest contributor to the overall burden of disease globally and in sub-Saharan Africa it is the second largest cause-specific source of disability adjusted life years (DALYs) (WHO 2002) after respiratory tract infections. Available evidence suggests that the incidence of fatal infections in Africa has been increasing since the early 1990s, most likely due to the emergence of widespread resistance to chloroquine and sulphadoxine/pyrimethamine (Bloland et al 1998). As mortality from poorly managed infections increases there is little hope for countries reaching the targets outlined in the Roll Back Malaria declarations at Abuja (WHO 2000).

In Zambia, disease estimates show that malaria rates have tripled in the past three decades to more than three million clinical cases per year in a total population of 10.3 million. Malaria accounts for 37% of all outpatient attendances (*Zambia Demographic Health Survey 2001/2002*). One of the major contributors includes a decline in the therapeutic efficacy of chloroquine and sulphadoxine/pyrimethamine (Bloland et al 1998, Chanda et al 2004), which has resulted into more expensive options for malaria treatment such as Artemisinin-based combination therapy (ACTs).

Vaccination as a control strategy is undermined by polymorphism in pathogen antigens which present a complex challenge for vaccine design (Kevin et al, 2005). *Plasmodium falciparum* exhibits extensive antigenic diversity. Although there is no universal strategy for the design of a vaccine against *Plasmodium falciparum* malaria, it is widely recognized that some of the existing diversity should be incorporated in the vaccine (Richie and Saul, 2002. Genton et al, 2002. However, the efficacy of any subunit vaccine containing a single form of MSP-2 may be limited by the presence of antigenically distinct parasite strains within an area of endemicity (Eisen et al, 1998, Smythe et al, 1990).

Extensive polymorphism of key parasite antigens is likely to reduce the effectiveness of subunit vaccines against *Plasmodium falciparum* infection (Eisen et al, 1998). There are

at least six antigenically diverse proteins of the merozoite that are known to be the target of potentially protective host responses expressed at all antigenically diverse loci, the extended antigenic haplotype (Eisen et al, 1998).

Sequence polymorphisms have been described for MSP-2 genes of both laboratory-maintained isolates and field isolates (Eisen et al, 1998, Smythe et al, 1990). However, little is known about the extent of the antigenic repertoire of naturally circulating genotypes in different areas where malaria is endemic in Zambia. Therefore, there is need to conduct a study to analyze *Plasmodium falciparum* MSP-2 parasite genotypes.

1.2   Statement of the problem

There is no information on *Plasmodium falciparum* MSP-2 genotypes encountered in the Zambian parasite population. These polymorphisms could limit the efficacy of subunit vaccines against *Plasmodium falciparum* infection if these variable proteins are not included in the candidate malaria vaccine.

1.3   Study justification

The Tropical Disease Research institution in Zambia is considering   setting up a vaccine trial site in Mpongwe a district in the Copperbelt and at the same time the malaria control programme in Zambia has embarked on deploying rapid diagnostic tests (RDTs) which only detect *Plasmodium falciparum* to be used in health facilities without microscopy facilities .The epitopes incorporated in the vaccine and used to manufacture these RDTs were not obtained from the Zambian parasite population. These two vital malaria case management and control activities need the latest information on *Plasmodium falciparum* genotypes encountered in the Zambian populations. The information generated in this study will be useful in the possible inclusion of the Zambian parasite epitopes as this has the potential to increase the efficacy of the vaccine and sensitivity of the malaria rapid diagnostics.

1.4   Literature review

Around 2.5 billion people, at least 40% of the world's population is at risk of contracting malaria in over 90 countries. Malaria contributes between 1.5 and 3 million deaths and up to 500 million acute clinical cases each year. Children account for the majority of these deaths, about 100 million in Sub-Sahara. Other high-risk groups include pregnant women, refugees, migrant workers, and non-immune travelers, (Eisen et al, 1998, Smythe et al, 1990).

Malaria is still a major public health problem in many tropical and subtropical countries. A Malaria vaccine is highly desirable as an adjunct to existing malaria control measures. The polymorphisms in malaria vaccine candidate antigens might be a hurdle in the development of an effective vaccine (Eisen et al, 1998). Variable regions of most genes are generally dimorphic, probably because of intragenic recombinations. Each allele in turn shows polymorphism resulting from point mutations, or other mechanisms (Simon et al, 2001). The merozoite surface protein 2 (MSP-2) of *Plasmodium falciparum* is extremely polymorphic. About 82 different MSP-2 alleles were found in four studies of molecular epidemiology conducted in Tanzania (Mbungi et al, 2006). This diversity renders MSP-2 suitable as a marker gene for the genotyping of *Plasmodium falciparum* infections. Amplification of MSP-2 by the polymerase chain reaction (PCR), and subsequent restriction digests of the PCR product (PCR-restriction fragment length polymorphism genotyping), has proved to be an informative tool for enumerating multiple concurrent infections in a blood sample, and distinguishing individual alleles (Simon et al, 2001,Felger et al, 1994).

Merozoite surface protein 2 (MSP-2), which is encoded by a single-copy gene, is a 45-to 52-kDa integral membrane glycoprotein anchored on the surface of the merozoite by a glycosylphosphatidylinosital (GPI) moiety. MSP-2 consists of highly conserved N (43 residues) and C (74 residues) terminal flanking a central variable region. This central variable region consists of centrally located repeats, which are flanked by none repetitive sequences. MSP-2 sequences are assigned to one of the two families, FC27 and IC-

1/3D7, based on the none repetitive sequences .The central repeats, which vary in number, length, and sequence among isolates, define individual MSP-2 alleles.

The degree of antibody reactivity to MSP-2 is sequence dependent, for example, antibodies that are inhibitory to parasites expressing a particular form of MSP-2 do not inhibit parasites expressing a different form (Anders et al, 1993). Field studies on parasite genomic DNA extracted from infected blood suggest that there is a large repertoire of circulating genotypes (Anders et al, 1993). Much of these data come from various PCR methods, such as restriction fragment length polymorphism analysis of PCR products and Southern hybridization using MSP-2-specific probes, and rely on size differences between repeat regions. Such methods underestimate the true level of diversity, which includes sequence differences due to mutations (Anders et al, 1993). Nucleotide sequencing provides a more accurate estimate of the antigenic micro heterogeneity of MSP-2. Several studies have examined MSP-2 alleles in field populations by nucleotide sequencing.

In areas of endemicity, immunity to *Plasmodium falciparum* malaria develops slowly and is hardly ever complete. One explanation for this phenomenon is that many different parasite strains, differing in the sequences of key protective antigens, circulate within any given area of endemicity (Felger et al, 1994). If immunity to infection were strain specific, then a state of generalized immunity would develop once exposure had occurred to a large enough sample of the many distinct parasite strains circulating in that region (Felger et al, 1994).

Thus, according to this explanation, the extensive degree of polymorphism noted in many surface antigens contributes to immune evasion and aids parasite pathogenesis. This polymorphism would also appear to restrict the effectiveness of subunit vaccines against P. falciparum infection if these variable proteins are included (Anders et al, 1993, Felger et al, 1994). Presumably, the extent of such subversion of the immune response would depend on the number of distinct antigenic forms circulating in an area of endemicity.

Since strain variation is an important component of immune evasion, vaccines incorporating variant proteins might have to include a full reportoire of variant forms in order to provide full protection against infection (Anders et al, 1993, Felger et al, 1994).

One main limitation to the development of a vaccine against *Plasmodium falciparum* is the antigenic diversity, which is related to *Plasmodium falciparum* polymorphism. Evidence indicates that antigen diversity limits the efficacy of acquired immunity based on strain-specific anti-parasite immunity, (Sakihama et al 2006, Renia et al, 1997, Healer et al, 2004).

Malaria RDTs offer great potential for rapid and accurate diagnosis of malaria infections, which could lead to prompt and appropriate treatment of the disease. Unfortunately, the genetic diversity within the antigens targeted by the antibodies used in the RDTs has the potential to affect their sensitivity (Baker et al, 2005).

Currently, RDTs target three major antigens of *Plasmodium falciparum* and these are *Plasmodium falciparum* histidine-rich protein (PfHRP) 2, *Plasmodium* lactate dehydrogenase (pLDH), and aldolase (Baker et al, 2005). In most studies, the sensitivity of RDTs have been found to be similar to that commonly achieved by microscopy (100 parasites/ μl) ,while in others the sensitivities were reported to be well below the level required for operational use (Baker et al,2005).

Factors that may affect the performance of malaria RDTs include parasite factors (species and level of parasitemia, variability in parasite antigen structure, and persistence of the antigen) and test factors (condition of the RDT, the technique used to perform the test, and the interpretation of the test results) (Baker et al, 2005).

One major factor is the variability within the parasite antigen being targeted by the RDT. This includes presence or absence of the target epitope or variation in the number of epitopes present in a particular parasite isolate (Baker et al, 2005). Genetic diversity may be particularly important for PfHRP2-based RDTs, since the antigen consists of a number

of alanine- and histidine-rich amino acid repeats and varies in size among parasite strains (Baker et al, 2005).

Comparison of the PfHRP2 sequences from several parasite strains show differences in the number of tri- and hexapeptide repeat units and rare amino acid variants and that the amino acid sequence of PfHRP2 for example in a Chinese isolate was different from that in South American (7G8) and Gambian (FCR3) isolates (Baker et al, 2005). In Zambia, however, no systematic examination of the diversity in PfHRP2 across geographically diverse regions has been performed, nor has any relationship between antigenic diversity and RDT detection sensitivity been examined. Therefore, this study was conducted in which blood samples on filter papers collected from parasitemic individuals living within endemic areas were analyzed to determine the extent of *Plasmodium falciparum* MSP-2 polymorphism and diversity in the Zambian parasite population.

1.5 Null hypothesis

Plasmodium falciparum parasites in Zambia do not exhibit genetic polymorphism and diversity.

1.6 General objective

The general objective of this study was to determine the distribution and degree of diversity of *Plasmodium falciparum* merozoite surface protein 2 (MSP-2) among symptomatic children aged five years and below in Zambia.

1.7 Specific Objectives

1. To determine the *Plasmodium* species isolated from the study sites.

2. To determine the prevalence of the MSP-2 genotypes of *Plasmodium falciparum* in Zambia.

3. To determine the frequency of MSP-2 *Plasmodium falciparum* genotypes

4. To determine the number of different genotypes in each infection of *Plasmodium falciparum*

CHAPTER: 2.0   MATERIALS AND METHODS

2.1   Study design

The study design was a prospective analytical study conducted for the first time in Zambia .The study involved further analysis of samples collected from children five years and below during the 2006 drug efficacy survey in the four selected sites in Zambia at the end of the rain season in April and may.

2.2   Study sites

Zambia has a diverse ecology that supports different *Plasmodium falciparum* transmission conditions. The following four districts were selected because they represent four major malaria ecologies.

(i)   Urban, hypo/mesoendemic area – Mansa District, Luapula Province

(ii)   Rural, semi-arid, mesoendemic area – Sesheke District, Western Province

(iii)   Mixed rural and urban, meso/hyperendemic area – Isoka District, Northern Province

(iv)   Rural, swampy, hyperendemic area – Mpongwe District, Copperbelt Province

In all these sites, malaria is endemic and transmission is perennial. These are sentinel sites for the antimalarial efficacy monitoring for Ministry of Health and represent all malaria epidemiological patterns in Zambia. Sites were selected from semi urban, urban and rural areas with high, medium and low malaria transmission patterns.

2.3   Study population

The blood smears and dried blood spots were collected from children resident in these sites, aged between zero and 5 years who attended the health facilities at the time of the 2006 Antimalarial efficacy monitoring.

2.4   Inclusion and exclusion criteria

All samples positive for *Plasmodium falciparum* by microscopy collected from the four sentinel sites were further analyzed. Blood samples from children aged between 0 and 5 years, residing in the four sites without the history of having traveled to other residences outside the sites in the past one month who presented with fever or history of fever were screened by the clinicians or Nurses were included in the analysis.

Blood samples from persons above 5 years old, without fever or history of fever, not residing in the four sites, or with the history of having traveled out side the four sites within one month and were negative for *Plasmodium falciparum* were excluded from the molecular analysis.

2.5   Sample size

The sample size was estimated to be 354 and was calculated using the formula $n=Z^2pq/d^2$.

Where;

N   =   sample size,

p   =   50 %(not known number of genotypes in Zambia)

q   =   46% (100-p), d=5(at 95% confidence level)

n   = $\underline{n=Z^2pq}$

d^2

n   = $\underline{(1.96)^2 \times 50 \times 46}$

$(5)^2$

n   =   353.4

$\approx$ 354

Table 1: Shows the total number of children screened for Plasmodium infections, number of positives blood smears examined to determine the species prevalence, number of dried blood spots collected, DNA extraction and PCR carried out by district.

Category	Mpongwe	Mansa	Sesheke	Isoka	Total
Total number of children five years and below screened for Plasmodium infection	354	404	256	206	1220
Total Positives to Plasmodium among the screened children five years and below	197	202	166	150	715
Number of dried blood spots collected for genotyping	93	61	70	61	285
Number of Patients from which dried blood spots were collected	46	27	35	32	140
Total number of dried blood spots sent for DNA extraction and PCR analysis	93	61	70	61	285
Total number of dried blood spots evaluable	2	10	17	3	32
Total number of dried blood spots with successful Parasite DNA extraction	91	51	53	58	253

A total of 285 dried blood spots on filter papers from 140 patients were collected for *Plasmodium falciparum* MSP-2 genotype determination from the four listed districts as shown in **table 1.**

2.6   Parasitological method

2.6.1   Microscopic examination, Thick and thin blood smears made from a finger prick in all the patients recruited during drug efficacy studies were re-examined under oil immersion (100 x magnifications) for the presence of *Plasmodium falciparum* parasites. All blood films were stained with Giemsa (3%). Parasites and leukocytes were counted in the same fields until 200+ or 500+ leukocytes were reached. Parasite densities were estimated using standard leukocyte count of 8,000 leukocytes/μL. Two independent microscopists read the slides to determine if they were positive or negative. Thin blood smears from patients detected to be positive for *Plasmodium* were re- examined and species were determined.

2.7   Molecular methods-(Appendix 1)

2.7.1   Dried blood spots These blood spots were made on filter paper Whatman NO. 3, air-dried at room temperature, individually wrapped in aluminum foil, put in plastic bags with a desiccant and kept at room temperature until the time for DNA extraction. They were collected before the patient was commenced on antimalarial treatment.

Only filter papers with thick blood smears positive to only *Plasmodium falciparum* were selected for molecular analysis.

2.7.2   Parasite genomic DNA extraction

Parasite DNA extraction was performed from dried blood spots with Chelex using Kain and Lanar (1991) modified method. The PCR micro well plate was used rather than the usual PCR micro tubes or strips. Samples were arranged in a specific order to which a map plate was created stating the position where each sample was placed on the micro plate. Using a sterilized pair of   scissors one leg of the serrated filter paper with a blood spot was cut and put on the mapped micro plate position. The pair of scissors was heat sterilized between the cuttings of different sample filter papers. A control filter paper without a blood spot was also included in one of the wells on each of the micro plate. To the cut filter papers in the micro plate wells, 200 micro litres of 0.5% saponin solution in Phosphate buffered saline (PBS) was added to each well using a mult-channel pipette. Precautions were taken to avoid pipette tips touching samples in the wells to avoid contamination. All the wells were capped and the micro plates incubated at 37^0C overnight in an incubator.

The 200 micro litres of saponin+ Phosphate buffered saline (PBS) +Hemoglobin were removed from each well and discarded. Each sample well was washed three times by adding and removing PBS from the wells after which 200 micro litres of freshly prepared 20% chelex suspension in water was added to each of the wells containing the sample. The top surface of the whole plate was sealed and on each well a hole was made using a

hot needle sterilized by heat between each well piecing. The whole plate was then incubated at 95^{0}C in a water bath for 5 minutes. At the end of the incubation period, the supernatants containing target DNA was removed and kept separately in a different matched micro plate frozen until it was needed for use.

2.7.3 PCR amplification of MSP-2 genes using family specific allelic primers

Before running all the samples, PCR methods were optimized and after desirable results were obtained the details of those methods which produced such results were written as protocols for sample analysis. Using these protocols, *Plasmodium falciparum* extracted DNA samples were analyzed for *Plasmodium falciparum* parasite diversity and polymorphism by a nested PCR amplification using oligonucleotide primers for the conserved regions of MSP-2 as well as oligonucleotides for specific family alleles for the variable regions. Primers for both primary and secondary reactions were ordered from the DNA/RNA Synthesis Core Facility of the Johns Hopkins University.

Primary PCR was carried out using primers M2-OF (356) for the forward extension and primers M2-OR (357) for the reverse extension for the conserved region. The primer sequences used are as shown below: M2-OF-5'-ATG AAG GTA ATT AAA ACA TTG TCT ATT ATA-3' and M2-OR-5'-CTT TGT TAC CAT CGG TAC ATT CTT-3'. But Secondary PCR was run with two separate family specific -allelic primers. FC27 family was determined by using M2-FCF (358) for a forward reaction and M2-FCR (359) for a reverse reaction and there specific sequences used are shown below respectively M2-FCF-5'-AAT ACT AAG AGT GTA GGT GCA RAT GCT CCA-3'(358) and M2-FCR-5'-TTT TAT TTG GTG CAT TGC CAG AAC TTG AAC-3'(359.

IC-3D7 family determinations were carried out using the following family specific primer sequences: M2-ICF-5'-AGA AGT ATG GCA GAA AGT AAK CCT YCT ACT-3' (360) as the forward primer and M2-ICR-5'-GAT TGT AAT CGG GGA TCA GTT TGT TCG-3' (361) as the reverse primer. Each sample was run with the two family specific allelic primers.

Sample volume: Amplification reactions for MSP-2 with family specific allelic primers was carried out using 3 micro litres of DNA from the extracted product for the primary PCR and 2 micro litres of PCR product from the primary round was used in the secondary amplification.

Preparation of the Master Mix: The master mix constituted of, 1.0 micro litres of dNTPs of the concentration 2.5 mili molar, 0.63 micro litres of each primer of the stock concentration of 10 micro mole and 0.24 Units of Taq DNA polymerase in a final reaction volume of 25 micro litres. Both primary PCR for the MSP-2 conserved region and nested PCR reactions for the variable regions were performed on the same thermal cycler under the same conditions.

Thermocycler Amplification Conditions: The reaction mixtures were denatured at 95 ^{0}C for 5 minutes, followed by 35 amplification cycles at 95 ^{0}C for 30 seconds, 58 ^{0}C for 1 minute and 68 ^{0}C for 2 minutes and a final elongation at 68 ^{0}C for 5 minutes. Allele-specific positive controls and DNA-free negative controls were included in each set of reactions and indicated that reactions worked and were free of contamination.

2.7.4 PCR-restriction fragment length polymorphism analysis of MSP-2 genotypes

Restriction fragment length polymorphism analysis was achieved by using a *Hinf*I digest of each nested PCR product to analyze MSP-2 genotypes. The digestion helped to determine allelic families, multiplicity and individual genotypes.

In the *Hinf*I digest 15 micro litres of nested PCR product was digested with the restriction enzyme *Hinf*I in a total reaction volume of 20 micro litres. The 15 micro litres of nested PCR product was combined with 3.4 micro litres of distilled water (dH$_2$O), 1.5 micro litres of 10 X buffer number three and 0.1 micro litres (0.1 U) of *Hinf*I enzyme. Since several samples were being tested and digested simultaneously, a master mix reaction of all components except the nested PCR product was prepared and calculated for 100 samples for a 96 well PCR micro plate to allow for pipetting inaccuracies. From the total volume of prepared master mix, 5.0 micro litres aliquots of the master mix were distributed to pre-labeled PCR micro plate and the respective nested PCR products

following the plate map were added to the exact position. The contents were mixed adequately by voltexing and then incubated at 37°C in an incubator overnight.

Product detection: Ten micro litres of each PCR product before and after digestion with HinfI enzyme was loaded on a 2.0% agarose gel with Ethidium bromide. Ethidium bromide-stained DNA bands were visualized under ultraviolet light using Eagle eye and pictures were taken, scanned and kept for manual analysis. Individual alleles were identified by looking at the fragment length position and by the corresponding allele-specific primers which were used. The sizes of the PCR products were estimated by using a 100 base pair DNA ladder marker.

2.8 Data recording

Data analysis was based on the visual inspection of amplified fragments separated by a 2.0 % agarose gel electrophoresis, both before and after digestion with *HinfI* enzyme. MSP-2 allele diversity and polymorphism were determined based on the number of fragments, size of each fragment, and allelic family to which each fragment belongs. Fragment sizes were estimated by comparing with molecular size standards of a 100 base pair DNA ladder. Each fragment was assigned to one of the following sizes in base pairs (bp): 1, 80 bp; 2, 100 bp; 3, 120 bp; 4,140 bp; 5, 160 bp; 6, 180 bp; 7, 200 bp; 8, 210 bp; 9, 220 bp; 10, 240 bp; 11, 260 bp; 12,280 bp; 13,300 bp; 14,320 bp; 15, 340 bp; 16, 360 bp; 17,380 bp; 18,380 bp; 19,400 bp; 20,420 bp; 21,440 bp; 22,460 bp; 23,480;bp, 24,500 bp; 25,520 bp; 26,540 bp; 27,560 bp; 28,580 bp; 29,600bp based on the presence of specific band length for the two allelic families. (Basco et al, 2000, Smythe et al, 1991, Smythe et al, 1990). The *Plasmodium falciparum* MSP-2 genotypes isolated on day zero and any other day of failure were compared and determined if it was a re-infection or recrudescence.

2.9 Ethical clearance

The UNZA School of Medicine Ethics committee reviewed and approved the study. All the samples further analyzed were collected from patients recruited for the 2006 drug efficacy survey by Ministry of Health. No patient names were associated with the results in this molecular analysis.

3.1. *Plasmodium* Parasite rates among screened children five years and below.

One thousand, two hundred and Twenty children aged five years and below were screened for *Plasmodium* infections and the parasite rate from the four sites was sixty nine percent. **Figure 1**, show that parasite rates varied by site ranging between sixty one and eighty three percent. Isoka, Sesheke, Mpongwe and Mansa shows the parasite rates of eighty three, sixty nine, sixty eight and sixty one percent respectively.
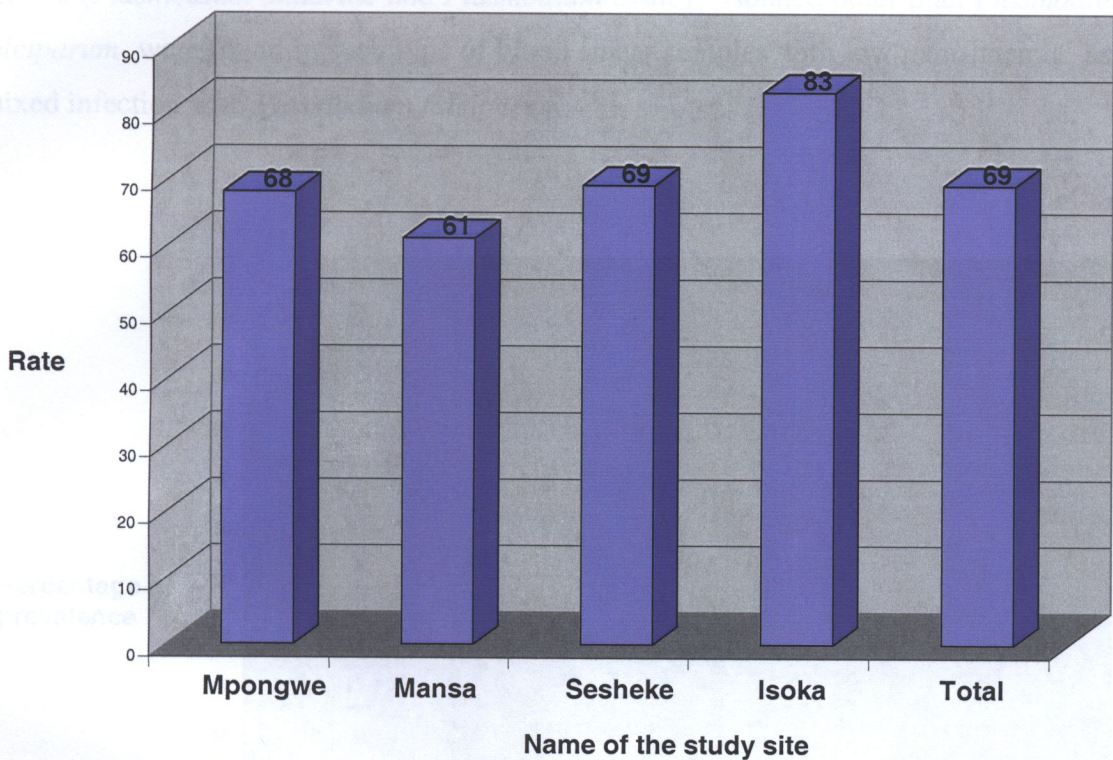
Figure1: Shows Plasmodium parasite rates obtained by examining thick blood smears stained in 3% geimsa using microscopy by district.

3.2. *Plasmodium* **species prevalence among screened children five years and below.**

Figure 2 shows the prevalence of different species for *Plasmodium* and it reveals that ninety eight point six percent of the blood slide samples were positive for *Plasmodium falciparum* while one percent and zero point four (0.4) percent blood smear samples were positive for *Plasmodium malariae* and *Plasmodium ovale* respectively. No *Plasmodium vivax* was detected from all the 715 total positive blood smear samples to *Plasmodium* parasites. The results also show that seventeen percent of the positive blood slide samples had less than 200 asexual parasites per 200+ leukocytes and both species (*Plasmodium malariae* and *Plasmodium ovale*) isolated other than *Plasmodium falciparum* were found in such type of blood smear samples with low parasitaemia as a mixed infection with *Plasmodium falciparum*.
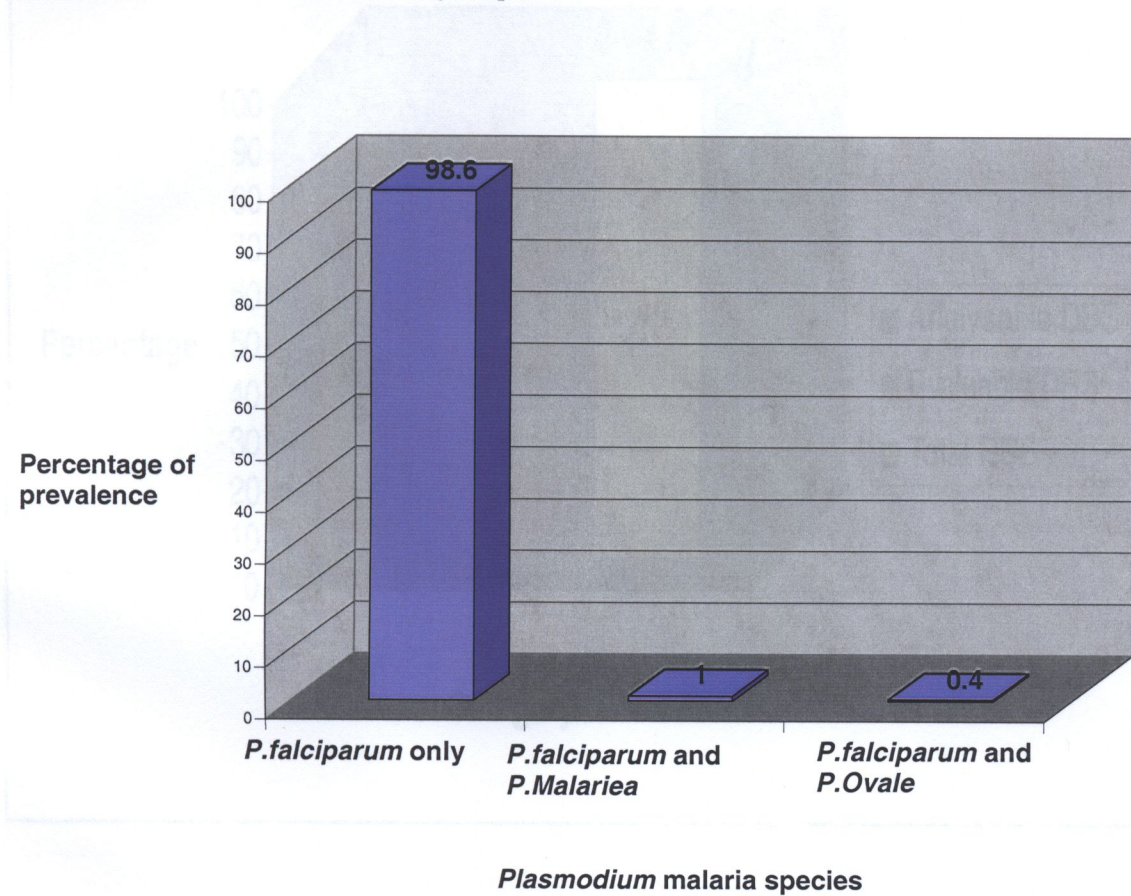


Plasmodium malaria species

Figure 2: The prevalence of different species of *Plasmodium* in children five years and below in the four study sites obtained by examining of thin blood smears stained in 3% geimsa

## 3.3. Parasite DNA extraction success rates from each of the four sites.

Two hundred and eighty five (285) dried blood spots were collected for *Plasmodium falciparum* DNA extraction. **Figure 3** shows that, eighty eight point eight percent of the dried blood spots collected successfully yielded enough *Plasmodium falciparum* DNA for analysis by PCR, while eleven point two percent did not yield targeted *Plasmodium falciparum* DNA. All the dried blood spots which failed to yield *Plasmodium falciparum* DNA were collected on the follow up days other than the day zero of the 28 patient follow up schedule.
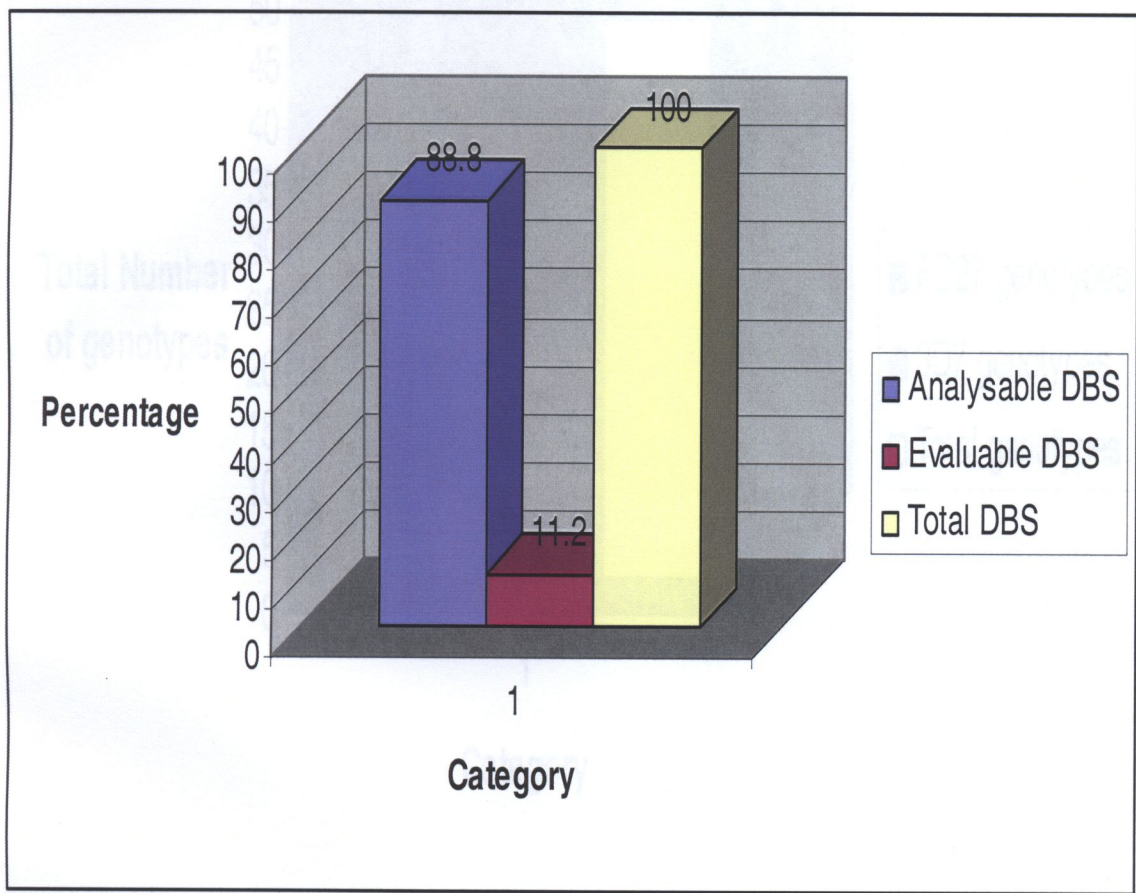
Figure 3: Percent of dried blood spots from which targeted Plasmodium falciparum DNA was successfully extracted using chelex extraction method.

3.4. Determination of the total number of *Plasmodium falciparum* MSP-2 genotypes among children five years and below in Zambia

The total number of circulating genotypes among the children aged five years and below in Zambia were found to be forty six ,with twenty four genotypes belonging to the FC27 family while twenty two to the 3D7 family as shown in figure 4.
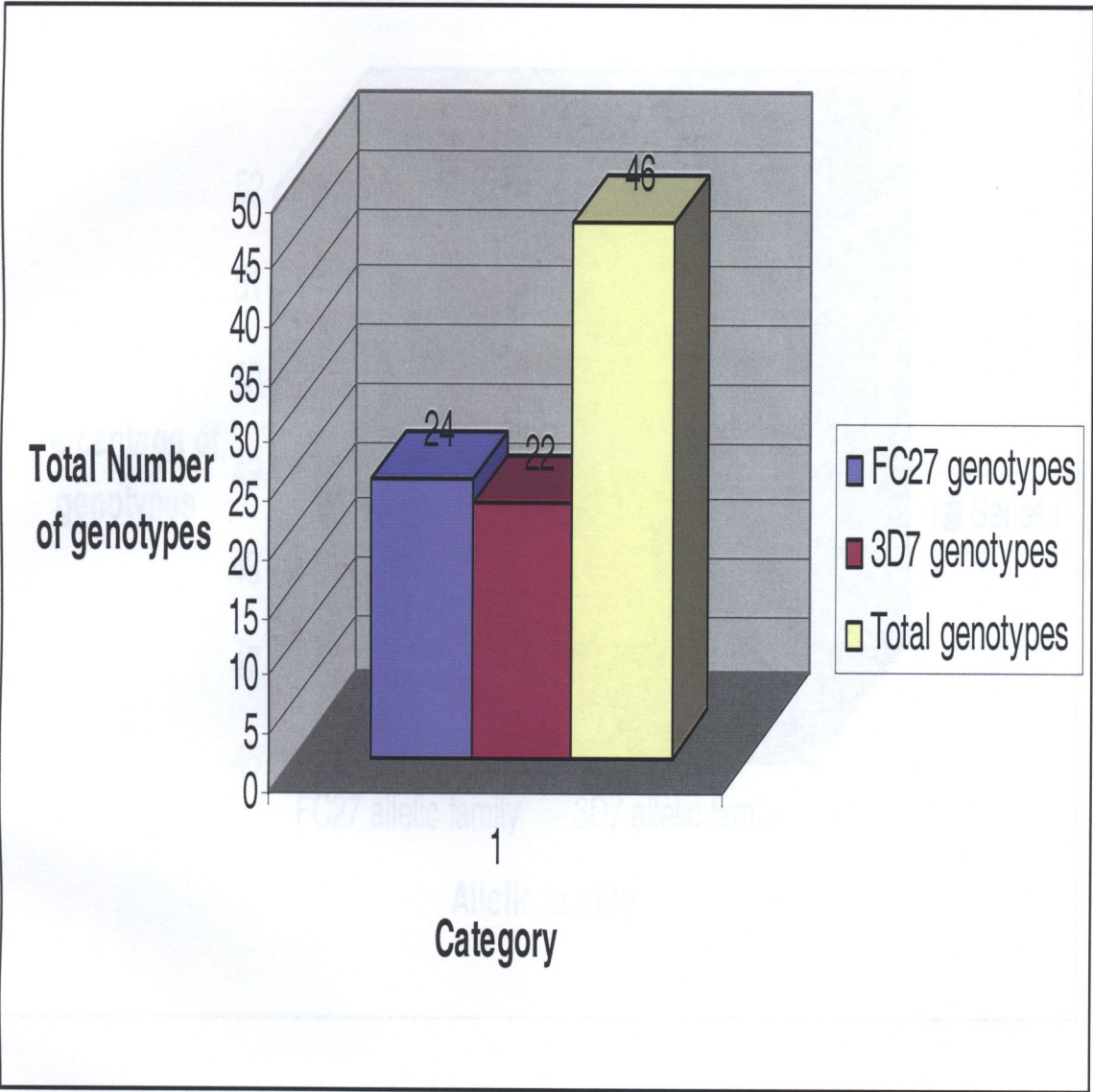
Figure 4: The total number of Plasmodium falciparum MSP-2 genotypes among the circulating parasites in the children aged five years and below in Zambia.

3.5. The most common MSP-2 allelic family of *Plasmodium falciparum*

Plasmodium falciparum can be grouped into two major allelic families, FC27 and 3D7 based on the length of the fragments in the conserved region of the parasite DNA. Figure 5 show that fifty two percent of the sampled children five years and below carried *Plasmodium falciparum* MSP-2 genotypes belonging to FC27 allelic family and forty eight percent from 3D7 *Plasmodium falciparum* allelic family.
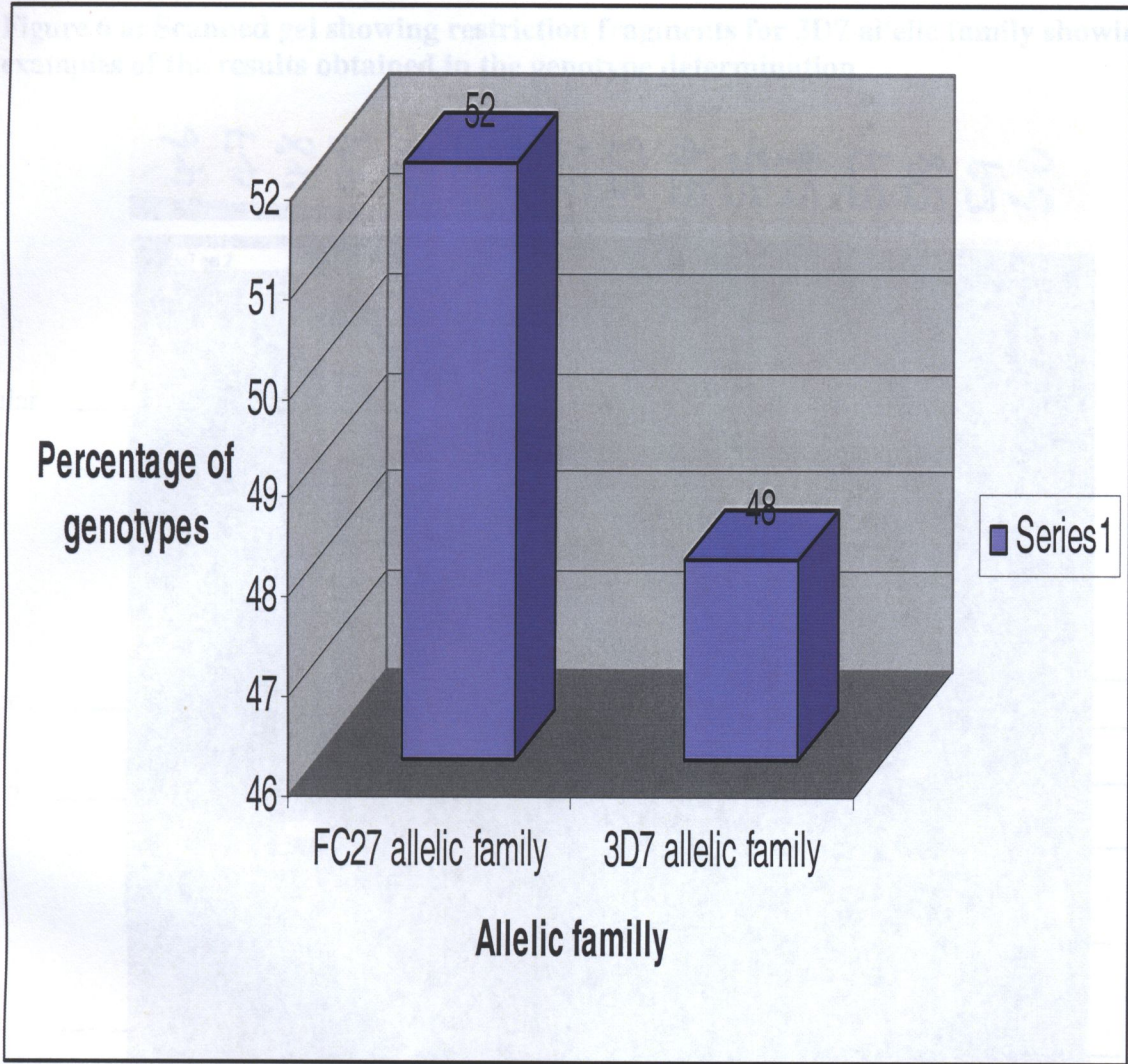

Figure 5: The percentage of dried blood spots from children aged five years and below carrying *Plasmodium falciparum* genotypes belonging to either FC27 or 3D7 allelic family.

3.6. Examples of scanned gels used in the interpretation of the results obtained in the MSP-2 genotype determinations.

Restriction digestion was used to determine the number of genotypes in each sample.
Figure 6 a ; shows that, Lane 46 and 59 carried no genotype in the 3D7 allelic family, but lane 47, 48, 52, 54, and 55 had single genotype infections. Lane 49,50,51,56 and 60 carried double genotypes whereas only lane 53 had triple genotypes in this allelic family.

Figure 6 a: Scanned gel showing restriction fragments for 3D7 allelic family showing examples of the results obtained in the genotype determination
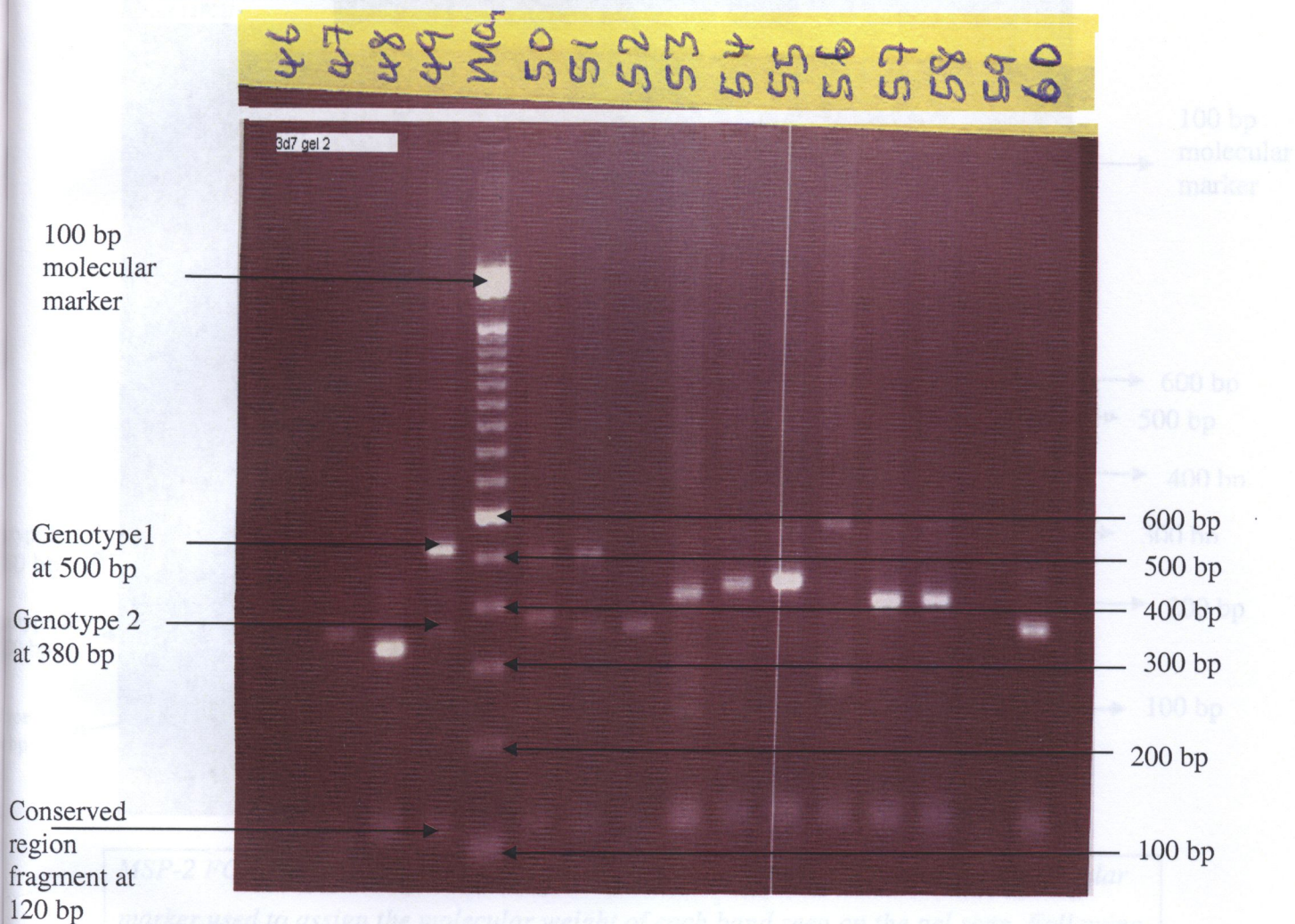
MSP-2 3D7 RFLP products on 2% agarose gel. Lane Ma is a 100 base pair molecular marker used to assign the molecular weight of each band seen on the gel scan. Following this method all the Plasmodium falciparum samples were analysed for the number of genotypes they carried in each of the allelic families.

Figure 6 b; shows that Example of the FC27 scans, line M representing the marker, while lines 1, 2 shows that the sample does not carry parasite genotypes belonging to this family. But lines, 3, 4, 5, 8, 9, 12, and 13 clearly shows that the samples carries parasites belonging to this family but are of different genotypes

Figure 6b: Scanned gel showing restriction fragments for FC27 allelic family showing an example of the gels obtained in the analysis
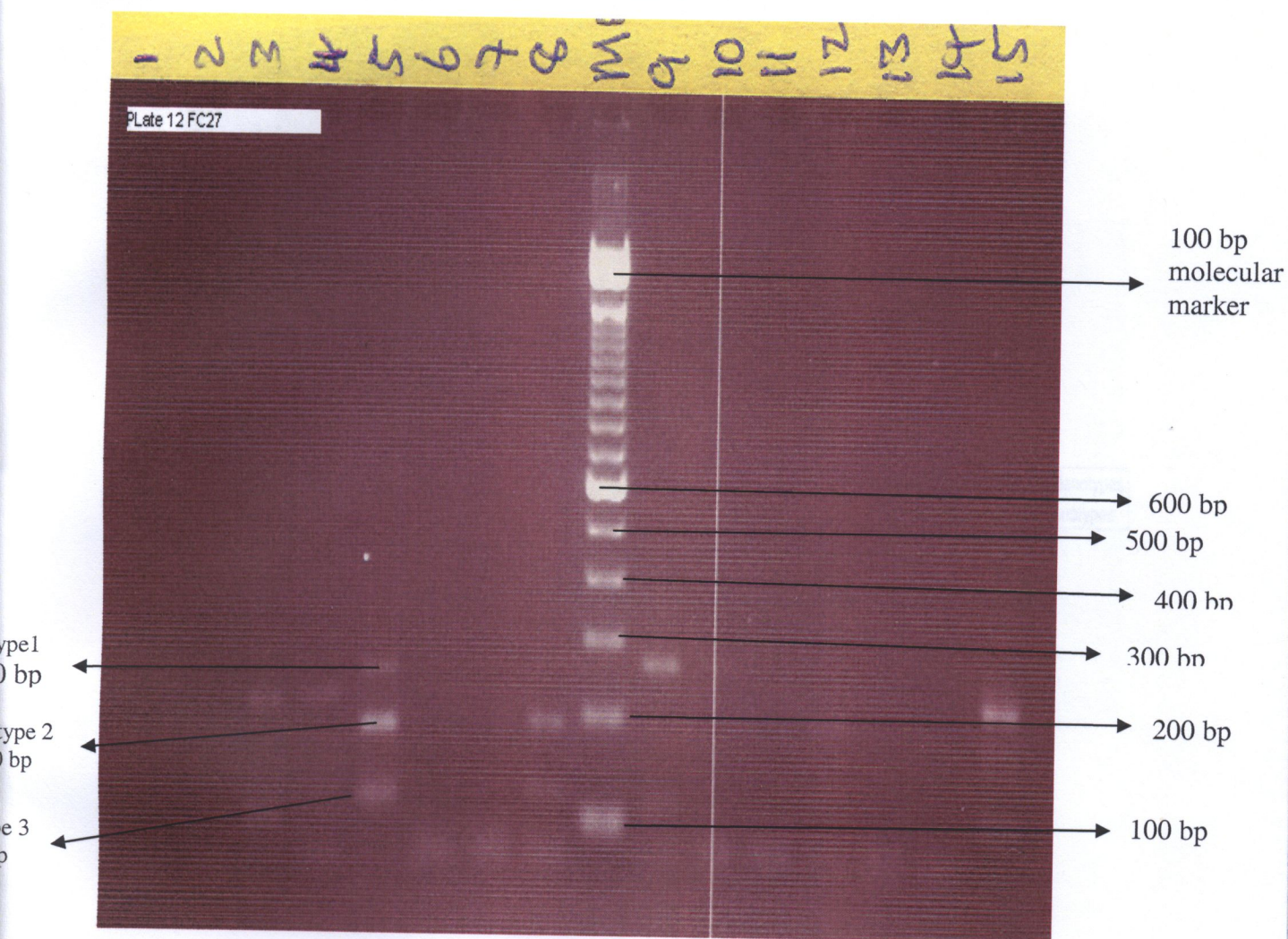

MSP-2 FC27 RFLP products on 2% agarose gel. Lane Ma is a 100 base pair molecular marker used to assign the molecular weight of each band seen on the gel scan. Following this method all the Plasmodium falciparum samples were analysed for the number of genotypes they carried in each of the allelic families.

3.7. Most frequent *Plasmodium falciparum* MSP-2 genotype by allelic family

The most frequent genotypes encountered among the children aged five years and below in the four study sites are 220 and 360 base pairs belonging to the FC27 and 3D7 allelic families respectively, followed by those with 280 base pairs in the FC27 allelic family. Figure 7 shows that the genotype of the base pair 360 belonging from the 3D7 allelic family is the most frequently encountered of them all.
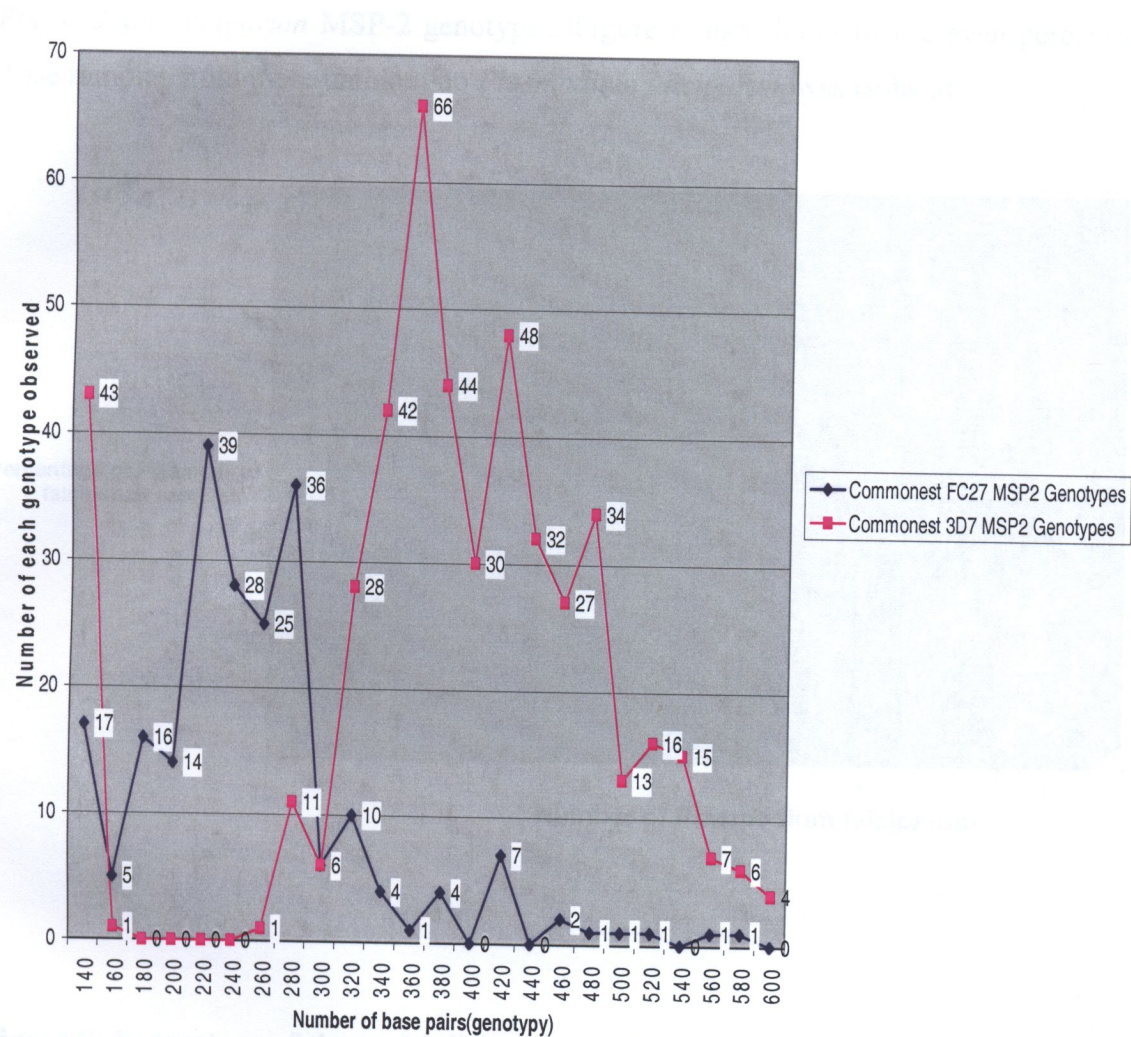

Figure 7: The most frequent *Plasmodium falciparum* MSP-2 genotype in the FC27 and 3D7 allelic families among the children aged five years and below.

3.8. Multiplicity of infection with *Plasmodium falciparum* MSP-2 genotypes

Figure 8 show that twenty one percent of the children aged five years and below infected with *Plasmodium falciparum* carried three different *Plasmodium falciparum* MSP-2 genotypes at a time in each infection. Four and one percent of these children carried seven and eight different *Plasmodium falciparum* MSP-2 genotypes respectively per infection. While fifteen percent of these children carried two and four different *Plasmodium falciparum* MSP-2 genotypes. Figure 8 also shows that, eleven percent of these samples from these children no *Plasmodium falciparum* was isolated.
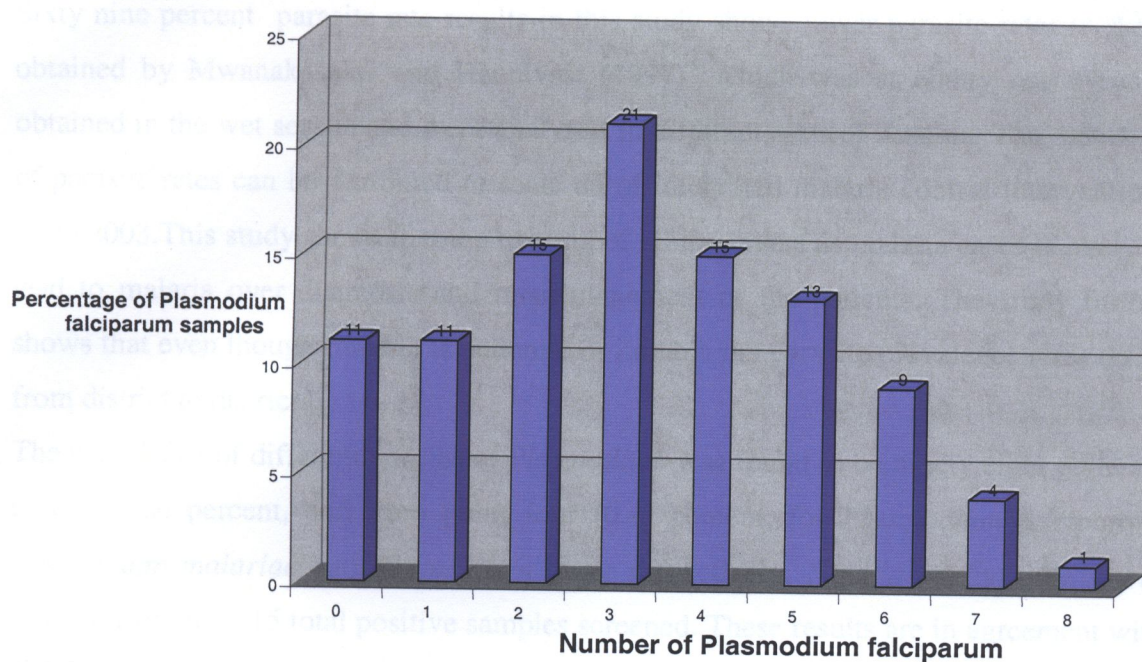

Figure 8: Percentage of the multiplicity of infection per infection with *MSP-2 Plasmodium falciparum* genotypes among the children aged five years and below

CHAPTER: 4.0 DISCUSSION AND CONCLUSION

The children five years and below recruited in this study, represent the age group most venerable to *Plasmodium falciparum* malaria (WHO, 2003). Four districts were sampled which represent four of the nine Provinces of Zambia.

4.1. Parasite rates and *Plasmodium* species prevalence

In this study, the *Plasmodium* parasite rate from all the four sites were sixty nine percent, but varied by site ranging between sixty one for Mansa to eighty three for Isoka. The Sixty nine percent parasite rate results in this study shows lower parasite rates to those obtained by Mwanakasale and Hauntvast (1997) which was at eighty one percent, obtained in the wet season and were analyzed in different sites of Zambia. The reduction of parasite rates can be attributed to scale up of integrated malaria control interventions since 2003.This study shows that the treating of all the fevers as malaria cases is likely to lead to malaria over diagnosis and mismanagement of the patients. The study further shows that even though malaria is endemic in Zambia the parasite prevalence rates differ from district to district.

The prevalence of different species of *Plasmodium* was found to be ninety eight point six percent, one percent, and Zero point four (0.4) percent, for *Plasmodium falciparum, Plasmodium malariae* and *Plasmodium ovale* respectively. No *Plasmodium vivax* was isolated from the 715 total positive samples screened. These results are in agreement with and further strengthen the policy being implemented in Zambia using the malaria rapid diagnostic tests that detect *Plasmodium falciparum* only to conform all malaria cases. With these findings that ninety eight percent of the malaria parasites are *Plasmodium falciparum* and that all the other two species that were isolated all coexist with *Plasmodium falciparum,* the fear of these malaria rapid diagnostic tests of giving false negatives due to other species prevalence are now no longer founded.
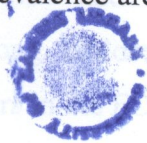
4.2. Successful PCR samples

The PCR amplification was carried out in 285 samples and was successful in ninety two percent and unsuccessful in eight percent. The PCR success and failures rates compare very well with those obtained by Mbugi et al (2006) who found similar results. Most unsuccessful PCR were obtained from follow up dried blood spots samples other than day zero (0) patient samples. The eleven point two percent unsuccessful PCR obtained in this study are probably due to some factors such as, failure to extract Parasite DNA, microscopy errors or sequestration of parasites at time the time of blood spot collection (Färnert et al., 1997), or due to point mutations at position where the MSP-2-specific primers anneal (Kassberger et al., 2002) thus impairing successful results.

4.3. Number of allelic genotypes

The study indicates that, both the 3D7 and FC27 alleles were prevalent in all the four study sites. Allelic diversity in the MSP-2 gene detected in this study was high with the 3D7 family showing 22 different genotypes while in the FC27 family 24 different genotypes were observed. The results in this study are in contrast with those reported by Smith et al. (1999) and Mbugi et al. (2006) in Tanzania in that both report a higher number of allelic genotypes from 3D7 family. In total forty six genotypes were isolated from among the children aged five years and below indicating that there is a great parasite diversity which needs to be incorporated in the antigen for the malaria vaccine. The existence of such a large poor of genotypes has some implications in the development of partial immunity to malaria as it is possible to be re-infected with totally a new pool of different genotypes to which the body has not yet developed partial immunity.

4.4. Most frequent Plasmodium falciparum MSP-2 genotype

The study shows that the genotypes with 220 and 360 base pairs are the most frequent ones in the allelic families FC27 and 3D7 respectively in the Zambian parasite populations isolated from among children aged five years and below. The next commonest was the 280 base pairs in the FC27 allelic family.

4.5. Multiplicity of genotypes for *Plasmodium falciparum* infections

Twenty one percent of the children in this study carried three different strains of Plasmodium falciparum per infection. In general seventy seven percent of these children carried two or more different genotypes at a time per infection. This means that there is possibility of these children carrying both sensitive and resistant strains to some antimalarials.

The number of concurrent genotype per blood sample (multiplicity of infection) in this study showed up to eight genotypes per *Plasmodium falciparum* infection with only one child harboring eight genotypes. These results show higher number of genotypes per blood samples than obtained by Mbugi et al (2006).

5.0   Conclusion

The findings have shown that there are forty six different MSP-2 genotypes of *Plasmodium falciparum* found in Zambia among children aged five years and below. It has also shown that *Plasmodium falciparum* is still the most prevalent species among all the other *Plasmodium* species and where other species occur they do as mixed infection with *Plasmodium falciparum* this justifies the use of the RDTs brand which only detect *Plasmodium falciparum*. In the study it was also clearly observed that *Plasmodium falciparum* infections carry more than one allelic genotype at each particular time, this means that it would help to consider the inclusion of all the available genotypes in both the malaria vaccine and RDTs for them to be of maximum benefit to the Zambian populations. This multiple infection with *Plasmodium falciparum* strains have other implications such as having one genotype sensitive to the drug and another resistant, therefore it needs to be further explored.

6. 0   References

Anders R. F., McColl D. J., and Coppel R. L., 1993. Molecular variation in Plasmodium falciparum polymorphic antigens of asexual erythrocytic stages. Acta Trop. 53:239-253.

Baker J., James M., Michelle G., Dennis E. K., Vicente B., Luchavez J., Bell D., and Cheng Q.,2005. Genetic Diversity of *Plasmodium falciparum* Histidine-Rich Protein 2 (PfHRP2) and Its Effect on the Performance of PfHRP2-Based Rapid Diagnostic Tests. The Journal of Infectious Diseases 192:870–7.

Bloland P.B., Kazembe P.N., Oloo A.J., Himoonga B., Barat L.M., and Ruebush T.K., 1998. Chloroquine in Africa: critical assessment and recommendations for monitoring and evaluating chloroquine therapy efficacy in Sub-Saharan Africa. *Trop Med Int Health* 3:543-552.

Chanda P., Sikaala C.H., Kapelwa W., Moonga H., Njunju E., Macdonald M., Thea D., Hamer D.H., and Sipilanyambe N., 2004. Assesment of the therapeutic efficacy of artemether-lumefantrine (Coartem®) and sulphadoxine-pyrimethamine (SP)-artesunate in Zambian children. *53rd Annual Meeting of the Society of Tropical Medicine and Hygiene, 7 – 11 November 2004, Miami, FL. Abstract 213.*

Eisen D., Billman-Jacobe H., Marshall V.F., Fryauff D., and Coppel R.L., 1998. Temporal variation of the merozoite surface protein-2 gene of Plasmodium falciparum,Infect. Immun., Jan 1998, 239-246, Vol. 66, No. 1.

Felger I., Tavul L., Kabintik S., Marshall V.F., Genton B., Alpers M., and Beck H. P.,1994. Plasmodium falciparum: extensive polymorphism in merozoite surface antigen 2 alleles in an area with endemic malaria in Papua New Guinea. Exp. Parasitol. 79:106-116.

Felger I., Irion A., Steiger S,, and Beck H.P., 1999. Epidemiology of multiple *Plasmodium falciparum* infections: Genotypes of merozoite surface protein 2 of *Plasmodium falciparum* in Tanzania. Transactions Roy. Soc. Trop. Med. Hyg. 93(1):3–9.

Genton B., Betuela I., Felger I., Al-Yaman F., Anders R. F., Saul A., and Rare L., Baisor M., 2002. A recombinant blood-stage malaria vaccine reduces Plasmodium falciparum density and exerts selective pressure on parasite populations in a phase 1-2b trial in Papua New Guinea. J. Infect. Dis. 185:820-827.

Healer J., Murphy V., Hodder A.N., Masciantonio R., Gemmill A.W., Anders R.F., Cowman A.F., and Batchelor A., 2004. Allelic polymorphisms in apical membrane

antigen-1 are responsible for evasion of antibody-mediated inhibition in Plasmodium falciparum. Mol. Microbiology 52: 159–168.

Kain K.C., and Lanar D.E., 1991. Determination of genetic variation within Plasmodium falciparum by using enzymatically amplified DNA from filter paper disks impregnated with whole blood. J. Clin. Microbiol 29: 1171–1174.

Kevin K., Tetteh A., Cavanagh D. R., Corran P., Musonda R., McBride J. S., and David J., 2005. Conway, Extensive Antigenic Polymorphism within the Repeat Sequence of the Plasmodium falciparum Merozoite Surface Protein 1 Block 2 Is Incorporated in a Minimal Polyvalent Immunogen13 April 2005.

Kassberger F., Birkenmaeir A., Khattab A., Kremsner P.G., and Klinkert M.Q., 2002. PCR typing of *Plasmodium falciparum* in matched peripheral, placental and umbilical cord blood. Parasitol. Res. 88:1073–1079.

Mbugi V.E., Mutayoba M.B., Sakurani T.B., Malisa L.A., Nyambo T.B., and Mshinda H.,2006. Multiplicity of infections and level of recrudescence in Plasmodium falciparum malaria in Mlimba,Tanzania.African Journal of Biotechnology,Vol.5(18), pp.1655-1662

Mwanakasale V., and Hauntvast., 1997, Mixed Plasmodium infection in rural children of Zambia. Cent Afr J Med. Jun; 41(6):186-7.

Renia L., Ling I.T., Marussig M., Miltgen F., Holder A.A., and Mazier D., 1997. Immunization with a recombinant C-terminal fragment of Plasmodium yoelii merozoite surface protein 1 protects mice against homologous but not heterologous P. yoelii sporozoite challenge. Infect. Immun. 65: 4419–4423.

Richie T. L., and Saul A.J., 2002. Progress and challenges for malaria vaccines. Nature 415:694-701.

Sakihama N.,Ohmae H., Bakotee B.,Kawabata M.,Hirayama K., and Tanabe K.,2006. Limited allelic diversity of Plasmodium falciparum merozoite surface protein 1gene from populations in the Solomon Islands,Am.J.Tropical Medicine and Hygiene ,74(1),2006,pp.31-40.

Simon W., Wang L., Billman-Jacobe H., Nhan D.H., Richie T.L., and Coppel R.L., 2001. Antibody Responses to Infections with Strains of Plasmodium falciparum Expressing Diverse Forms of Merozoite Surface Protein 2, Infection and Immunity, February 2001, p. 959-967, Vol. 69, No. 2.

Smith T., Beck H.P., Mwankusye S., Felger I., Fraser-Hurt N., Iron A., Alonso PL., Teuscher T., and Tanner M., 1999. The epidemiology multiplicity of *Plasmodium falciparum* infections 3. Age dependence of the multiplicity of *Plasmodium falciparum* infections and other malariological indices in an area of high endemicity. Transactions Roy. Soc. Trop. Med. Hyg. 93(1):11–14.

Smythe J. A., Peterson M .G., Coppel R. L., Saul A. J., Kemp D .J., and Anders R. F., 1990. Structural diversity in the 45-kilodalton merozoite surface antigen of Plasmodium falciparum. Mol. Biochem. Parasitol. 1990; 39:227–234.

World Health Organization. *World Health Report 2000.* Geneva, Switzerland, World Health Organization.

World Health Organization. 2003. Improving access to Antimalarial medicines. Report of the RBM partnership meeting. *[WHO/CDS/RBM/2003.44]. 2002.* Geneva, Switzerland, World Health Organization.

World Health Organization (WHO) 2003. Microbiology and Immunology: S2024: Malaria. [http: wwwmicro.msb.le.ac.uk/224/malaria.html] site visited on 13th June 2003.

Zambia. 2002. *Demographic Health Survey 2001/2002.* Lusaka.

7.0 Appendices

Appendix I: Reagent preparations

A. Phosphate buffered saline (PBS)
Add
- 11 gm of sodium phosphate dibasic.
- 2.5 gm of potassium phosphate monobasic.
- 2 gm of potassium chloride.
- 80 gm of sodium chloride.
- Dissolve all these in 10 litres of q water.
- Autoclave.

B. Saponin 0.5%
- Weigh 0.5 gms of saponin powder
- Dissolve it in 100mls of PBS

C. Chelex 20%
This is prepared just before use.
- Weigh 20gms of chelex crystals.
- Dissolve them in 100 mls of distilled water
- In a 200mls container with screw cap.
- Shake the mixture for 1 hour.
- Let it stand for 10 minutes
- Filter through a filter N.Y.0.2 micro mole
- The filtered chelex water is the one to be use.

D.10X TEA Buffer (Tris Acetic acid EDTA buffer)
Add
- 242gms of Tris base
- 29 gms EDTA
- 55 mls of glacial acetic acid
- q5 litres of water.
- Autoclave

E.1X TEA buffer
Add
- 1 part of 10X TEA buffer
- 9 parts of distilled water.

F. Preparation of a loading 6 X Loading Dye
- As per manufacturer s instructions

G.Preparation of a 100 bp DNA ladder 50 micro litres

- As per manufacturer s instructions
- Add 83 micro litres of 6 X loading buffer.
- Add 367 micro litres of Distilled water
- Equal to 500 micro litres of ready to use DNA ladder.
- Use 5 micro litres of DNA ladder per line.

2. DNA extraction
1. Arrange samples in the order you want.
2. Create a plate map, i.e. stating the position where each sample will be on the PCR plate.
3. Using a sterilize pair scissors cut one leg of the filter paper with blood spotted on it.
4. Heat sterilize the pair of scissors between the cuttings of different samples or filter papers.
5. Put each cut filter paper as indicated on the plate map.
6. Add 200 micro litre of 0.5% saponin to each well using a mult-channel pipette.
7. Take care not for the pipette tip to be touching the samples in the wells.
8. Cap the wells.
9. Incubate the plate at 37degrees celcious overnight in an incubator.

The following morning

1. Remove the 200 micro litres of saponin+PBS+Heamoglobin.
2. This can be discarded or kept for other analysis.
3. Wash the samples three times with PBS by adding and removing the PBS from the wells.
4. Add 200 micro litres of prepared 20% chelex solution (water to each of the wells containing the sample.
5. Incubate the mixtures at 95 degrees celcious in a water bath for 5 minutes.
6. Remove the suppernant and keep it for PCR in separate plate.
7. The plates with DNA must be kept frozen until needed for use.

3. PCR Methods

A. Optimization of PCR protocol
All the methods need to be optimized before put into full scale use to see if the results are desirable.

B. Preparation of the master mix

	Components	X1	X 33
1	10 X PCR buffer	05.0mL*	165.0mL
2	dNTPs(A,T,C,G mixture)	01.0mL*	033.0mL
3	Primer F1 (forward)	01.0mL*	033.0mL
4	Primer R1 (reverse)	01.0mL*	033.0mL
5	Taq polymerase	00.20mL*	06.6mL
6	Distilled water	39.8mL*	1313.4mL
	Total	48.0mL*	1585.0mL
7	Sample DNA	02.0mL*	
	Final reaction volume	50.0mL*	

*mL= micro litres
- It is a good practice to first add distilled water.
- Add Taq. Polymerase last in the master mix just before you are ready to dispense into the micro plate wells.
- It is advisable not to take long before PCR is started after adding all reagents.
- Optimization result will determine the out come of the PCR for other tests. If the results from the optimization are desirable then your are ready to run all the samples without changing anything as was done in the optimization methods.

Determination of *Plasmodium falciparum* Parasite Diversity Using MSP-2 Family-Specific Allelic Primers among Symptomatic Children Aged Five Years and Below in Zambia

Moonga Hawela[1], Cecilia Shinondo[2], Sungano Mharakurwa[3], David Sullivan[4]

[1]National Malaria Control Centre, Ministry of Health, Lusaka, Zambia; [2]University of Zambia School of Medicine, Lusaka, Zambia; [3]Malaria Institute at Macha, Macha, Zambia; [4]Johns Hopkins University, Baltimore, MD, United States
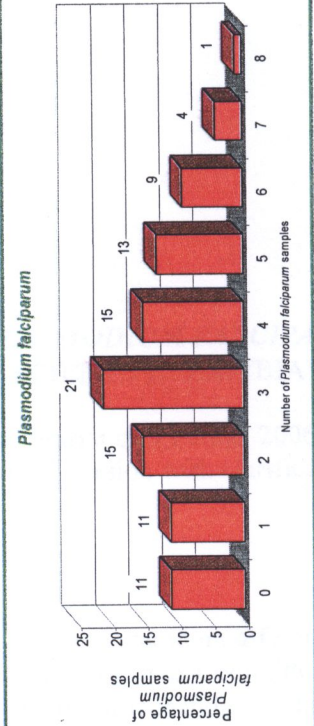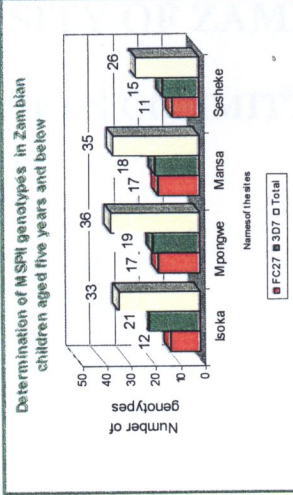
Introduction: Malaria is still a major public health problem in many tropical and subtropical countries. Therefore, effective integrated malaria control strategies are a requirement. However, parasite antigen diversity might be a hurdle in the efficacy of some malaria control strategies like the malaria vaccine against *Plasmodium falciparum* infection if a full repertoire of variant forms is not incorporated into the vaccine. Multiple *P. falciparum* infections will have other implications such as having one genotype sensitive to the drug and another resistant to the same drug. Therefore, there was a need to investigate *P. falciparum* parasite diversity in Zambia.
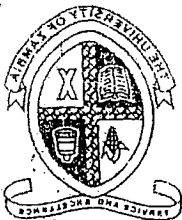
Methods: In 2006, a prospective analytical study was conducted to determine the diversity of *P. falciparum* in four sites in Zambia. Nested polymerase chain reaction (PCR) amplification was carried out on two hundred and eighty-five dried blood spots with *P. falciparum* mono infection using primers for the conserved regions of MSP-2 as well as family-specific alleles for the variable regions. MSP-2 amplicon was subjected to restriction fragment length polymorphism analysis using a *Hinf*I digestion, followed electrophoresis on ethidium bromide-stained 2.0% agarose gels to determine the allelic families and genotypes.

Results: The study findings show that there are forty-six different genotypes of *P. falciparum* commonly found in Zambia: twenty-four allelic family, and twenty-two are from the 3D7 allelic family. The study shows that twenty-one percent of the *P. falciparum* samples carried three different *P. falciparum* MSP-2 genotypes. Four percent and one percent carried seven and eight different *P. falciparum* MSP-2 genotypes, respectively. Fifteen percent of these samples carried two and four different *Plasmodium falciparum* MSP-2 genotypes.
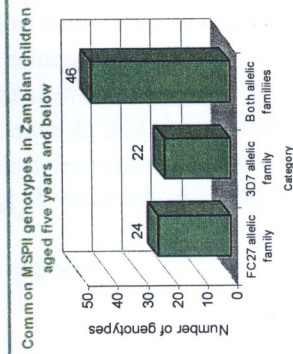
Discussion and Conclusions: These findings show that *P. falciparum* in Zambia exists with a high genotypic diversity, and most patients infected with *P. falciparum* carry at least more than two genotypes. The impact on detection and spread of drug resistance needs to be explored with the scale-up of interventions such as effective drug therapy and bednets.

Determination of MSPII genotypes in Zambian children aged five years and below

Plasmodium falciparum

Common MSPII genotypes in Zambian children aged five years and below

THE UNIVERSITY OF ZAMBIA

RESEARCH ETHICS COMMITTEE

Telephone: 260-1-256067
Telegrams: UNZA, LUSAKA
Telex: UNZALU ZA 44370
Fax: + 260-1-250753
E-mail: unzarec@zamtel.zm
Assurance No. FWA00000338
IRB00001131 of IORG0000774

Ridgeway Campus
P.O. Box 50110
Lusaka, Zambia

4 September, 2006
Ref.: 012-08-06

Mr Moonga B. Hawela
National Malaria Control Centre
P.O. Box 32509
LUSAKA

Dear Mr Hawela,

RE: RESEARCH PROPOSAL ENTITLED: "DETERMINATION OF *PLASMODIUM FALCIPARUM* SPECIES, GENOTYPE DIVERSITY AMONG SYMPTOMATIC PATIENTS IN ZAMBIA"

The above research proposal was presented to the Research Ethics Committee Secretariat on 29 June, 2006 where changes were recommended. We would like to acknowledge receipt of the corrected version with clarifications. The proposal has now been approved. Congratulations!

CONDITIONS:

- This approval is based strictly on your submitted proposal. Should there be need for you to modify or change the study design or methodology, you will need to seek clearance from the Research Ethics Committee.
- If you have need for further clarification please consult this office. Please note that it is mandatory that you submit copy of your final report at the end of the study.
- Any serious adverse events must be reported at once to this Committee.
- This approval is granted on the understanding that the proposed microscopic examination will be carried out on blood samples already collected during the drug efficacy studies, and that specimens exported to the USA will not be used for any other purpose.
- The need to obtain informed consent is already waived.

Yours sincerely,

Prof. J. T. Karashani, MB, ChB, PhD
CHAIRMAN

Date of approval: 4 September, 2006   **Date of expiry:** 3 September, 2007

JHMRI

JOHNS HOPKINS
MALARIA RESEARCH
INSTITUTE

February 20, 2007

Dr. Moonga Hawela
Biomedical Scientist (Medical Scientific Officer)
National Malaria Control Centre
Zambia

Dear Dr. Hawela:

   We were very pleased to have you do an endemic scientist rotation with us and hoped that your training will help you throughout your career. The benefits of such training, helps not only the participant but the people here at Johns Hopkins Malaria Research Institute. You have completed all required guidelines, the final being the acceptance and approval of your progress report. We look forward to continued collaboration with you and hope that with your new training, that you will be able to develop future collaborations with colleagues. If we can be of further assistance to you, do not hesitate to contact me or our JHMRI office at 410-502-3377 Susan Booker (sbooker@jhsph.edu) or 443-287-4853 Maryann Brooks (mbrooks@jhsph.edu).

Sincerely,

Nirhbay

Nirhbay Kumar
Professor
Johns Hopkins University-Bloomberg School of Public Health
Department of Molecular Microbiology & Immunology
JHMRI

malaria.jhsph.edu

JOHNS HOPKINS
BLOOMBERG

JOHNS HOPKINS
BLOOMBERG
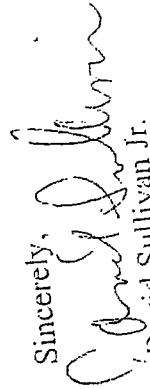SCHOOL of PUBLIC HEALTH

August 1, 2006

Moonga Hawela
Biomedical Scientist (Medical Scientific Officer)
National Malaria Control Centre
Zambia

RE: Acceptence for Visiting Scientist Fellowship

Dear Moonga,

Congratualtions on your acceptance to our endemic scientist training program where you
will be a visiting scientist for three months to learn about malaria control. As your sponsor we
will provide room and board living expenses while you are visiting and also purchase your
airfare. We expect you to arrive late August staying until end of November or another three
month period that suits your schedule.

Sincerely,

David Sullivan Jr.
Associate Professor
Johns Hopkins Bloomberg School
Of Public Health. Molecular
Microbiology & Immunology
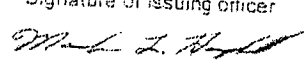And also Infectious Diseases

DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE

Centers for Disease Control and Prevention
Office of Health and Safety, MS E-79
Atlanta, Georgia 30330
TEL 404-498-2260 FAX 404-498-2275
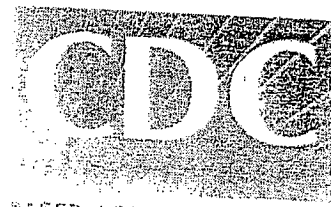
Permit to Import or Transfer Etiological Agents or Vectors of Human Disease

In accordance with 42 CFR Section 71.54 of the Public Health Service Foreign Quarantine Regulators, cited on the bottom of this permit, permission is granted the permittee to import into any port under control of the United States, or to receive by transfer within the United States, the material described in Item 1 below

SAFER · HEALTHIER · PEOPLE

PHS PERMIT NO.	2005-07-016	
DATES	ISSUED: Tuesday, July 05, 2005	EXPIRES: Wednesday, July 05, 2006
1 DESCRIPTION OF MATERIAL	BLOOD, URINE, SALIVA, SERUM AND BONE FROM HUMANS THAT MAY CONTAIN PLASMODIUM FALCIPARUM, PLASMODIUM MALARIAE, PLASMODIUM VIVAX, OR PLASMODIUM OVALE.	
2 PERMITTEE (NAME, ORGANIZATION, ADDRESS)	DAVID SULLIVAN JOHNS HOPKINS BLOOMBERG SCHOOL OF PUBLIC HEALTH 615 N. WOLFE STREET BALTIMORE, MD 21205	TEL: 410-502-2522 FAX: 410-955-0105
3. SOURCE OF MATERIAL (NAME, ORGANIZATION, ADDRESS, COUNTRY)	PHILIP THUMA MALARIA INSTITUTE AT MACHA BOX 6300166 CHOMA, ZAMBIA	

4 TYPE OF PERMIT AND INSTRUCTIONS FOR USE

☑ Multiple Importation into the US   ☑ Multiple Transfer Within the US

A. Record of each importation shall be maintained on permanent file by permittee

B. Enclosed label(s) must be forwarded to the shipper(s).

C. One label shall be affixed to shipping container. Enclosed labels may be photocopied.

5 CONDITIONS OF ISSUANCE

ITEMS APPLICABLE WHEN CHECKED

☐ A. Subsequent distribution, within the U.S., of the material described in this permit is prohibited without prior authorization by the Public Health Service

☑ B. All material is for laboratory use only - Not for use in the production of biologics for humans or animals.

☑ C. All material is free of ... serum and plasma of domestic and wild ruminants, swine, and equines.

☐ D. Additional Requirements

    File APHIS/CDC Form 2 for select agents as defined in 42 CFR 73

    IATA Packaged to preclude escape.

    USDA permit may be required (Telephone: 301-734-3277).

☑ E. Work with the agent(s) described shall be restricted to areas and conditions meeting requirements in the CDC/NIH publication "Biosafety in Microbiological and Biomedical Laboratories"

☑ F. Packaging must conform to 42 CFR Section 72 and 49 CFR Sections 171-180.

☐ G. Select Agent. Receiving facility must be registered under 42 CFR Part 73

6 COPY SENT TO

U.S. QUARANTINE STATION

7 Signature of issuing officer

Mark L. Hemphill

Mark L. Hemphill, M.S., Office of Health and Safety

9.28 (F 13-40) REV 2-91

CFR 71.54 Etiological agents, hosts, and vectors

A person may not import into the United States, nor distribute after importation, any etiological agent or any arthropod or other animal host or vector of human disease, or any exotic living arthropod or other animal capable of being a host or vector of human disease unless accompanied by a permit issued by the Director.

Any import coming within the provisions of the section will not be released from custody prior to receipt by the District Director of the U.S. Customs Service of a permit issued by the Director

Other permits may be required.