HIV/MALARIA CO-INFECTION: EFFECT OF HIV INFECTION ON ANTIMALARIAL TREATMENT OUTCOMES IN CHILDREN IN ZAMBIA

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Master of Science in Medical Parasitology

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DECLARATION

This dissertation is the original work of MUTENGO MWALE MABLE. It has been done in accordance with the guidelines for MSc. in Medical Parasitology dissertations of the University of Zambia. It has not been submitted elsewhere for a degree at this or another University.

Signature.....

Date 02/07/08

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I...MABLE MUDALE - MUTENGO.....

Hereby certify that this dissertation is the product of my own work and, in submitting it for my Master of Science in Medical Parasitology Programme, further attest that it has not been submitted to another University in part or whole for the award of any Programme.

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I, Dr.....

Having supervised and read this dissertation is satisfied that this is the original work of the author under whose name it is being presented.

I confirm that the work has been completed satisfactorily and is ready for presentation to the examiners.

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CERTIFICATE OF APPROVAL

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ABSTRACT

Children co-infected with HIV-1 and malaria are more likely to suffer from malaria and may respond poorly to anti-malarial treatment due to immunosuppression resulting from HIV infection. This study provides results of antimalarial treatment outcomes in children co-infected with HIV and malaria.

The study was a health facility based case control study and was conducted between December 2006 and October 2007.

One hundred and twenty four children were recruited from five study sites with stable and unstable malaria transmission patterns. Among the 124 children recruited, 9 had HIV-1 and malaria co-infections (cases) while 113 were non-HIV-1 and malaria infected (controls). Treatment was according to the Zambian government malaria treatment policy guidelines; thus Quinine for complicated malaria and Artemether Lumefantrine (Coartem) for uncomplicated malaria. All the children recruited were followed-up for 28 days. Eighty children completed the day 28 follow-up. Clinical assessment and malaria parasite examination were done on each day of the visit while samples for PCR were collected on day 14 and any day thereafter if the patient was malaria positive by blood slide. Molecular genotyping was used to distinguish re-infection parasites from recrudescent parasites. Data was analyzed with SPSS version 11.0. Chi-square test was used to test for significant differences of baseline data among the two groups and the difference in means was measured with the unpaired t-test. Tests of significance could not be performed on treatment outcomes because of the low numbers of patients in the case group.

Out of the 80 children who completed day 28 follow-up, 8 were cases and 72 were controls. The mean haemoglobin levels among children in the case and control groups at baseline was 6.62 ±2.71 and 9.55±2.40 respectively (p-value <0.05). The parasite count geometric means for the case and control groups were 11501 and 7550 respectively. The mean CD4 counts for the children in the case group 356±138.47 while the control had 1171.86±445.18 (p-value <0.05). Fifty percent of the cases presented with complicated malaria upon recruitment as compared to 6.9% in the controls. A total of 16 post

treatment positive samples were recorded of which 4 were positive by both microscopy and 12 were positive by PCR only. In order to distinguish re-infection parasites from recrudescent, all the 16 positive post treatment samples were genotyped. Out of the 16 post treatment malaria positive samples recorded, 5 (31.3%) were due to re-infections and 10 (62.5%) were recrudescents. Recrudescent parasites were seen in 6.2% and 62.5% of the cases and controls respectively. Treatment failure rates within the case and control groups were 1 (12.5%) and 10 (13.8%) respectively.

Our study has documented higher parasitaemia and prevalence of severe malaria among HIV-1 malaria co-infected children.

However, the study findings have shown that the risk of developing treatment failure is less likely among children with severe immunosuppression as seen in HIV malaria infected children. These results may not be significant and conclusive due to a number of factors including the low prevalence rates of HIV and malaria co-infections and small study sample size.

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Dedication

To my Husband and my two children (Chongo and Tinkho) for their great support and understanding they gave me during my work.

Chapter	Table of contents	Page

LIST OF TABLES

LIST OF ABBREVIATIONS AND SYMBOLS

ACPR.....Adequate Clinical and Parasitological Response

AIDS.....Acquired immune deficiency Syndrome

AL.....Artemether Lumenfantrine

CTF.....Clinical Treatment Failure

DHMT.....District Health Management Team

DNA .....Deoxyribonucleic Acid

EDTA..... Ethylenediamine Tetraacetic Acid

ETF.....Early Treatment Failure

FBC.....Full blood count

HIV.....Human immune Virus

LPF.....Late Parasitological Failure

LTF.....Late Treatment Failure

MSP .....Merozoite surface protein

NMCC.....National Malaria Control Centre

PBS ..... Phosphate buffered saline

PCR..... Polymerase Chain Reaction

SP.....Sulphadoxine-Pyrimethamine

TBE.....Tris Borate EDTA

UNAIDS.....Joint United Nations Programme on HIV/AIDS

UNZA.....University of Zambia

UTH.....University Teaching Hospital

UV.....Ultra Violet Light

WHO.....World Health Organization

<.....Less than

>.....Greater than

%.....Percentage

±.....Plus or Minus

≥.....Greater or equal to

≤.....Less or equal to

CHAPTER ONE: INTRODUCTION

1.1 Background

Malaria and Human immune deficiency Virus (HIV) infection are highly prevalent in Sub Saharan Africa and are of great public health concern (WHO, 2004). There are 300-500 million cases of malaria infection occurring worldwide and about 90% of these cases are caused by *Plasmodium falciparum* which occur mainly in sub-Saharan Africa. This results in approximately 1 million deaths, mostly in children under five years of age (Obonyo *et al,* 2004). *P.falciparum* accounts for 95-97% of all malaria infections in Zambia with *P ovale* and *P malariae* accounting for less than 2% of the total malaria infections (NMCC, 2004).

UNAIDS estimated the number of HIV infections worldwide to be 37.8 million by the end of 2003, of which 25 million were estimated to occur in Sub-Saharan Africa (NAC, 2003). Acquired Immune Deficiency Syndrome (AIDS) claimed the lives of an estimated 2.4 million people and over 600 000 were newly infected with the virus in 2003 (WHO, 2004). HIV/AIDS accounts for a large proportion of mortality among children less than 5 years of age in heavily affected countries (WHO, 2004).

Malaria and HIV/AIDS are mainly diseases of and causes of, poverty and they share determinants of vulnerability (WHO, 2004). Both diseases are responsible for much suffering and are a burden to economies of most affected countries as time, resources and manpower are directed towards ways of trying to control them in order to improve the health status of the majority poor people. Co-infection with malaria and HIV is common where the two diseases coexist in the general populations or in specific high-risk groups (WHO, 2004). The extensive geographic overlap and high prevalence of these two infections in Sub Saharan Africa and the potential to interact with each other is of considerable public health concern (Rowland- Jones and Lohman, 2002; WHO, 2004). The increased prevalence of malaria and malaria parasite density among HIV infected individuals could result in increased malarial transmission in both the HIV and non HIV individuals. The

increase in the risk of clinical malaria among HIV infected people could result in increased burden on the already struggling clinical services in areas with high HIV and malaria prevalence (Whitworth, 2004).

Host immune responses against malaria infections are important in determining the outcome of the disease. Acquired immunity is neither permanent nor completely protective but rather is associated with low grade parasitaemia and episodes of disease throughout life (Perlmann, 2002). Immunity to malaria infection develops early in life but children below the age of 5 years and pregnant women are at high risk of increased malaria morbity and mortality than immunocompetent adults (Whitworth, 2005).

In areas of stable malaria transmission, children born to immune mothers are protected against disease during their first half year of life by maternal antibodies. This passive immunity is lost during the first year of life and is followed by 1or 2 years of increased susceptibility before acquisition of active immunity (Perlmann, 2002).

Acquisition of active immunity to malaria is slow and requires repeated parasite exposure in order to be maintained. Most antimalarial drugs are effective in the presence of a good immune response in the elimination of malaria parasites and if HIV infection impairs the immune response to malaria, co-infection could result in decreased antimalarial drug efficacy (White N.J, 1998; Obonyo, *et al* 2004).

In pregnant women, HIV infection is likely to impair the ability of these women to control *P. falciparum* infection. It is estimated that in 2003 in sub-Saharan Africa at least 440 000 women had malaria infection during pregnancy because of HIV (WHO, 2004). Most women in their first or second pregnancy are at higher risk of severe or complicated malaria than during subsequent pregnancies. HIV alters this typical pattern by shifting the burden from mainly women in their first or second pregnancy to all pregnant women. Co-infected pregnant women are at increased risk of anaemia, preterm birth and intrauterine growth retardation as compared to women with either malaria or HIV infection. As a result, a considerable proportion of children born to women with dual malaria and HIV infection have low birth

weight and are more likely to die during infancy. The presence of HIV results in a poorer response to both prophylaxis and treatment of malaria during pregnancy (WHO, 2004).

1.2 STATEMENT OF THE PROBLEM

In Zambia, malaria is one of the major public health problems and is the leading cause of morbidity and mortality, especially in children under the age of five years (NMCC, 2004). It accounts for 31.8% and 35.6% of hospital and other health care facilities attendances, respectively. Malaria also accounts for about 40% of all outpatient attendances (NMCC, 2004).

According to estimates by the NMCC, malaria in Zambia has been accounting for nearly 4.3 million clinical cases and approximately 50,000 deaths per year. In heavily affected countries, Zambia inclusive, HIV/AIDS accounts for a large proportion of mortality among children less than 5 years of age (WHO, 2004). Although there has been increase in knowledge and understanding on HIV and malaria interactions, limited data are available on the patterns of treatment responses among children with HIV co- infected with malaria.

1.3 STUDY JUSTIFICATION

In this study, we sought to further elucidate the effect of HIV infection on antimalarial treatment outcomes, whether indeed it has negative effects on antimalarial response in children who are co-infected with malaria. Having a clear understanding of these interactions may help health planners to develop policies and strategies that will be directed towards improving malaria case management in HIV and malaria co-infected children thus reducing morbidity and mortality due to these two diseases.

CHAPTER: TWO LITERATURE REVIEW

2.1 Literature Review

Adequate clinical and parasitological response to antimalarial drugs is most effective in individuals who have acquired some immunity to malaria (Shah *et al* 2006). It would therefore be hypothesized that response to antimalarial treatment will be reduced in immunosuppressed HIV and malaria co- infected individuals living in regions of stable malaria transmission. Since the emergence of the HIV pandemic, a number of studies have been conducted in order to elucidate the interactions that may exist in HIV and malaria co-infections. Findings from these studies however, have produced contradicting results especially where studies have been conducted in children. Early investigations conducted in the Democratic Republic of Congo concluded that there were no differences in response to antimalarial therapy in HIV and malaria co- infected children compared with HIV negative children with malaria and no difference in parasitaemia in the two groups (Nguyen-Dinh *et al* 1987; Greenberg *et al* 1991). A negative association found between HIV infection and malaria parasitaemia was seen in a study by Villamor *et al* (2003). Lower prevalence of malaria among HIV infected as compared with uninfected children was also noted.

A cohort study that was conducted in an area of stable malaria transmission in rural Uganda found that HIV and malaria co-infected adults were more likely to have higher levels of parasitaemia compared to those without HIV infection. The study also showed that decreasing CD4 levels and advancing HIV disease were risk factors for higher parasitaemia and increased malaria episodes among these individuals (Whitworth *et al,* 2000). These results correlated well with studies conducted in Malawi and South Africa showing that advancing HIV disease with higher HIV-1 RNA concentration and low CD4+ T cell count resulted in higher Parasitaemia and increased episodes of fever (Grimwade *et al*, 2004; Patnaik *et al*, 2005).

Chirenda and Murugasapillay (2003) reviewed literature in order to assess the association between malaria and HIV/AIDS co-infection for the purposes of developing strategies for malaria control. Their conclusion was that HIV-1 infections increased the incidence of *Plasmodium falciparum* parasitaemia and was associated with development of severe malaria in HIV-1 malaria co-infected people as compared to non HIV-1 malaria infected people. This, therefore, calls for more concerted efforts in designing specific and appropriate intervention measures that will improve the management and control of malaria infections among HIV and malaria co-infected people.

Grimwade *et al* described associations among HIV status, presentation and outcome from malaria in children and adults in an area of high HIV prevalence and unstable *Plasmodium falciparum* transmission. Their conclusion from the two studies was that HIV infection among these patients was a risk factor for severe malaria, coma and even death (Grimwade *et al* 2003; Grimwade *et al* 2004). Chirenda *et al* also documented similar findings in adult Zimbabweans co-infected with HIV and malaria. In their study, they concluded that HIV infection was significantly associated with the development of severe and complicated malaria. The risk of developing complicated malaria among HIV and malaria co-infected patients was 2.35 (95% CI 1.85 to 2.98) times more than in the HIV negative malaria patients (Chirenda *et al*, 2000).

Several recent studies have also studied the effect of HIV infections on antimalarial treatment outcomes and that most of these studies have indicated that antimalarial drugs may be less efficacious in people living with HIV. Results from a large cohort in Uganda indicates that treatment with sulfadoxine pyrimethamine (SP) and artemether-lumenfatrine (Coartem) is less effective in HIV non pregnant malaria infected adults (Kamya *et al,* 2006). The study further showed an increased risk of clinical treatment failure (CTF) among HIV seropositive adults with genotyping results suggesting new infections rather than recrudescences as compared to non

HIV infected adults. Clinical treatment failure by day 28 after treatment was 20 % for the HIV and malaria co-infected adults and 7 % in non HIV adults with malaria (Kamya *et al,* 2006). However, in this same study, the risk of clinical treatment failure was the same in HIV and malaria co- infected children and in the non HIV children infected with malaria.

Levels of immunity in an individual play a role in the way they respond to antimalarial treatment as was seen in a randomized controlled study conducted in Zambia among HIV and malaria co- infected adults (Van Geerturuyden *et al* 2006). The study found that HIV-1 infected adults co-infected with malaria and having CD4 cell count less than 300 cells/µl had a higher risk of experiencing recrudescent infection than those with a CD4 cell count of 300cells /µl or higher. These findings are similar to those conducted by Shah *et al* (2006) who demonstrated that HIV infected adults with low CD4 counts and anaemia had a 3.4 times increased hazard of treatment failure occurring within 28 days of receiving SP therapy for treatment of uncomplicated malaria, compared with those who were non- HIV infected presenting with malaria (Shah *et al* 2006).

Birku *et al* (2002) investigated the effect of artemisinin on the rate of clearance of *Plasmodium falciparum* in patients with or without HIV. The study showed a higher mean clearance time in the HIV seropositive group (37.7 hr ± 2.2) as compared to the HIV seronegative group with clearance time of 30.0 hr ±2.1. The study further showed a 12 fold higher mean parasite density in the seropositive group than in the seronegative group.

Laufer *et al* (2006) however, on the other hand showed that decreased CD4 was not associated with treatment failure as earlier reported by the above studies. Among children, the risk of treatment failure increased with infection with SP-resistant parasites and anaemia. Decreased CD4 cell count was not associated with impaired response to antimalarial therapy or diminished ability to clear SP-resistant parasites,

suggesting that acquired immunity to malaria is retained in the face of HIV-associated immunosuppression (Laufer *et al* 2007).

Kashamuka *et al* (2003) did not find any significant correlation between HIV Status and dysfunction of immune response against malaria infection in adults in the Congo DR. These findings contradict with findings from other studies that have shown significant association between HIV and malaria infection in terms of increased parasitaemia, malaria episodes and reduced antimalarial treatment response (Obonyo *et al,* 2004; Whitworth *et al,* 2000). The study did not measure levels of immunity in the HIV infected individuals. The lack of correlation could have been attributed to high levels of immunity among this group if the study was conducted in the early stages of HIV infection.

In Sub-Saharan Africa, HIV and malaria are leading causes of morbidity during pregnancy. Results from studies in HIV- malaria co infected pregnant women have shown more placental and peripheral malaria infections, higher parasite densities and more febrile illness, severe anaemia and adverse birth outcomes than in non HIV malaria infected pregnant women (Ladner *et al*,2002; Feiko *et al,* 2004). In this background of conflicting results and non conclusive findings on the impact of HIV-Malaria interactions more studies are necessary to further elucidate the various unclear issues. In this study we, thus, sought to assess the antimalarial treatment outcomes of children Co-infected with HIV and malaria.

2.3 HYPOTHESIS

2.3.1 HIV infection is not associated with increased malaria severity in children.

2.3.2 Immunosuppression in HIV infected children is not associated with reduced treatment response to antimalarial therapy.

The above hypotheses are based on the assumption that HIV infected children are more likely to suffer severe malaria and respond poorly to antimalarial treatment.

2.4 GENERAL OBJECTIVE

The general objective of this study was to determine the effect of HIV infection on malaria severity and treatment outcomes in children.

2.5 SPECIFIC OBJECTIVES

The specific objectives for this study were:

2.5.1 To determine the presence of malaria parasites rates in blood and estimate levels of parasitaemia

2.5.2 To determine levels of immunity in HIV- malaria co-infected children and non-HIV children with malaria using CD4 cell counts.

2.5.3 To determine anaemia levels in HIV- malaria co-infected children and non-HIV children with malaria.

2.5.4 To determine the efficacy of standard antimalarial treatment in both HIV – malaria co-infected children and non HIV children with malaria.

2.5.5 To distinguish recrudescent from re-infection malaria parasites using molecular genotyping as a means of confirming treatment outcomes in HIV and malaria co-infected children and non-HIV children with malaria

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study Design

The study was a health facility based case control cohort study and was conducted between December 2006 and October 2007.

2.2 Population

The study population comprised children from 2 to 14 years presenting with body temperature higher than 37.5°C or with history of fever in the last 24 hours and a positive malaria slide. All children with positive malaria slides were recruited into the study after having obtained consent from their parents or guardians. HIV testing was performed upon recruitment and obtaining consent from parents or guardians. Thus, patients with positive malaria slides and HIV- positive were recruited into the study as cases and those with positive malaria slides and HIV negative served as controls.

2.3 Study sites

The study was conducted at the University Teaching Hospital Department of Paediatrics and Child Health in Lusaka, Chongwe district referral Health Centre (Chongwe District), Mpongwe Mission Hospital (Mpongwe District) and Mpulungu urban health centre the in Northern Province of Zambia. All study participants were recruitment from the outpatient departments of the five study centres.

2.4 Inclusion Criteria

Children 2 years - 14 years of age with confirmed malaria (clinical Diagnosis with Positive blood slide), history of fever preceding the past 24 hours, body temperature of 37.5 or higher with a positive blood slide and no history of antimalarial treatment in the preceding 4 weeks were included into the study. Parents or guardians who consented to have their children participate in the study had their children included.

2.5 Exclusion Criteria

Children with known immunodeficiency disorders or chronic disease other than HIV, on long term immunosuppressive therapy such as steroids and those on antimalarial treatment in the previous 4 weeks were excluded from participating into the study.

2.6 Sample Size

The study sample size for the case and the control groups were calculated employing the following formula (Dobson, 1981);

$$n = \frac{P1\,(100-P1) + P2\,(100-P2)}{(P1-P2)^2} \times f(\alpha, \beta)$$

Where;

n= the sample size

P1= proportion in the control group

P2 = proportion in the case group

α = significance level

1- β = Power required (Dobson 1984)

Having 20 % clinical failure (CTF) proportion in HIV infected adults and 7% CTF in non HIV infected adults (Kamya *et al*), the sample size for the study at 80% confidence interval and 0.05 significance level using a two- tailed test was worked out as follows;

$$n = \frac{7\,(100-7) + 20\,(100-20)}{(7-20)^2} \times 7.85$$

=105

Thus 105 case group and 105 control group giving a total of 210.

At the time this sample size was being calculated, no prevalence rates for treatment failures among children and adults were available in Zambia. In addition, and more importantly, there was no local data on the incidence of children co-infected with

HIV and malaria that subsequently was found to be relatively low. This resulted into difficulties in recruiting required sample size for the cases.

2.7 Parasitological determination of presence of malaria parasites and density

Malaria parasite rates and parasite densities were determined in all children who were recruited into the study. Blood was collected onto clean well labelled glass slides and thick and thin films were made. Thick films were dehaemoglobinized by dipping in tap water for a few seconds and stained with 10% freshly prepared Geimsa solution for 15 minutes. The thin smear was fixed in methanol for a few seconds before staining in 10% Giemsa for 30 minutes. The stained smears were air dried and examined for malaria parasites under a microscope using x100 oil immersion objective. Thick smears were used to detect the presence of malaria parasites and parasite species identification was performed using thin smears.

A slide was declared negative after 100 fields were counted and no parasite was seen. The slides were read by two independent scientists and were there was a discrepancy on two results a slide was re-read by a scientist. In order to estimate the malaria parasite density, parasite per microlitre of blood method was used on thick smears. It is based on the assumption that 8 000 leucocytes are found in a microlitre of blood.

Thus:

Number of Parasites X 8 000= parasites per µL

Number of leucocytes

Two tally counters were used to count parasites and leucocytes separately and the readings were recorded in a log book.

If 200 leucocytes were counted and 10 or more parasites identified, the number of parasites was per 200 leucocytes. If after 200 leucocytes were counted and the parasites were 9 or less, then 500 leucocytes were counted and the number of parasites was per 500 leucocytes (WHO, 1991). Malaria parasite density estimations were performed by two independent laboratory scientists and the

average result from the two readings was recorded. In the event that the two readings had discrepancies, the readings were repeated.

2.8 Determination of HIV Serostatus and immunity levels

Samples from cases and controls were tested for HIV- 1 antibodies upon recruitment using two methods. Two different HIV antibody detection kits Determine ½ (Abbot Diagnostics) and Genie 11 (Bio-rad) were used to determine HIV sero-status of children upon recruitment. The tests were run in parallel and patients were classified as HIV positive if the results of both tests were positive and HIV negative if the results from both tests were negative. All indeterminate results were repeated. The tests where performed according to the manufacturer's instructions. Positive and negative known samples served as controls for this study.

All samples for CD4 cell count determination were collected in EDTA bottles and were run within the same day of collection on a Facscount machine. The method for running this test was according to the manufactures instructions which was strictly followed and adhered to (BD BIOSCIENCES). In order to maintain quality on the results produced commercially prepared controls were run before the samples could be analysed.

2.9 Determination of haemoglobin levels

Samples for haematological studies from the case and control groups were collected in EDTA anticoagulant containers and were analyzed within 6 hrs of collection. Haemoglobin levels were obtained from the Full blood count results run on the ABX Micros analyzer according to the manufacturer's instructions. In order to ensure that quality results were produced, an internal quality control was performed using commercially prepared controls before the samples were run. Samples that produced unsatisfactory results where repeated.

2.10 Standard antimalarial treatment efficacy evaluation

Even though, many studies have been done on the effect of HIV infection on malaria presentation, very few studies have examined the effect that HIV infection has on antimalarial treatment response in children co-infected with malaria. This evaluation therefore, was aimed at establishing whether indeed reduced immune response as seen in HIV infections results in impaired response to antimalarial treatment.

All malaria positive patients recruited to the study were treated with either Quinine or Coartem depending on the presentation of malaria and according to the malaria treatment policy in Zambia. Children with complicated and uncomplicated malaria were treated with Quinine and Coartem respectively. The children were followed up for a period of 28 days as follows; Day 0, Day 3, Day 7, Day 14, Day 21 and Day 28 (WHO 1996, WHO 2003). Clinical assessment and malaria parasite examination was performed on all the follow up days. Dry blood spots were collected on day 14, and any day there after if a slide was positive for malaria parasites.

2.11 Distinguishing recrudescent from re-infection malaria parasites

Infection with HIV-1 may result in an impaired immune response to malaria by increasing cellular immunesuppression, with higher likelihood of increased parasitaemia and clinical illness (Obonyo *et al,* 2004). This assessment in the study was aimed at confirming whether repeated clinical illness and presence of malaria parasites after treatment is due to re-infection or recrudescence.

In order to distinguish true recrudescences from re-infections, pre and post treatment samples positive by microscopy or PCR on day 14 and any day there after were genotyped using molecular methods (Snounu and Berk, 1998; Snounu, 2002).

(i) Specimen collection

Pre and post treatment blood was collected from malaria positive patients on days 14, 21, and 28 onto Schleicher and Schuell grade 903 filter papers. The blood spots were dried and put in individual envelops and stored at room temperature until further analysis. The spots were clearly labelled with the patients study number and date of collection before being stored away.

(ii) Genomic parasite DNA extraction.

Parasite DNA was extracted from dried blood spots with Chelex using Kain and Lanar modified method (Kain and Lanar 1991). Dried sample strips were inserted into the mouth of a pre-labeled 1.5 ml eppendorf tube by making a right angle fold at the blood free base. Using a pair of scissors the blood free section was cut allowing the blood section of the filter paper to drop into the tube. The pair of scissors was cleaned with 70% alcohol and passed through a bunsen burner in between cuttings to avoid cross contamination of the samples.

After the samples were loaded into the eppendorf tubes 1ml of autoclaved 1X phosphate buffered saline saponin solution (0.5g saponin in 100mls autoclaved PBS) was added to each of the tubes and left at room temperature for 10 minutes or until solution turned brown. The sample tubes were spun at 14,000 rpm in a micro centrifuge for 3 minutes. The supernatant and any debris were aspirated with a micropipette using a different pipette tip with each sample leaving the strips in the tube. Another 1ml autoclaved 1X phosphate buffered saline was added to each tube and samples were spun again at 14,000 rpm for 3 minutes. The supernatant was discarded leaving the sample strips in the tube. To each of the tubes, 150µl of autoclaved double distilled water and 50µl of 20% chelex resin suspension (20g chelex resins in100ml double distilled water) in autoclaved double distilled water was added. The tubes were then closed and a fine hole on top of the lid was made with a sterile heated hypodermic needle. Sterile needles were used for each tube. The sample strips in the chelex resin were boiled at 97°C in a heating block for 8 minutes. After boiling the samples were spun at 14,000 rpm speed for 2 minutes.

The supernatant (~100µl) was transferred into two PCR tubes and kept at 4° C and -20°C respectively pending amplification.

(iii) Detection of Malaria Parasites using PCR

Microscopy still remains the gold standard for the diagnosis of malaria parasites and parasitological follow up of malaria patients in most countries. However, this method has limitations in that its sensitivity decreases with decreasing levels of parasitaemia (Snounu 2002). A nested PCR therefore, was performed in order to detect malaria parasites missed by microscopy method on subsequent post treatment days. All dry blood spots collected on the follow-up days were used to confirm negative blood slides after DNA extraction as described above.

PCR amplification methods were optimized before amplification was performed. Template DNA was amplified using the following primer sequence for the first reaction (nest 1):

r PLU1 TCAAAGATTAAGCCATGCAAGTGA

CCTGTTGTTGCCTTAAACTTC r PLU5

Amplification was performed in a 20µl reaction volume. The master mix was fully thawed and vortexed before being aliqouted into individual well-labeled PCR tubes. A 2X stock master mix (Fermentas) comprising 0.05 u/µl *Taq* DNA polymerase reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP (dATP, dCTP, dGTP, dTTP) used. The final concentration of each of the components in the master mix at a reaction volume of 20µl per tube was 2 Mm MgCl, 1 unit of *Taq* Polymerase, 125µM of each of the dNTPs and 2µl of DNA template.

Nest 1 amplification conditions were as follows: Step 1, 95° C for 5minutes; step 2, 58°C for 2 minutes; step 3, 72°C for 2 minutes; step 4, repeat steps 2-4 for a total of 25 cycles; step 6 58°C for 2 minutes; step 7, 72°C for 5 minutes and step 8, the reaction was complete and the temperature was reduced to 4°C (Snounu 2002).Amplification was performed using a Gene Amp PCR thermal cycler 2700 (Applied Biosystems, CA).

The amplicon from the first reaction (Nest 1) served as the template for the second reaction (nest). Reagents used in Nest 1 were used and the final concentration at a 20µl reaction volume was the same. One microlitre of the nest one product was added to the master mix aliquoted into each of the pcr tubes. Nest 2 amplification conditions were similar to those in nest one except that the annealing temperature in step 3 was 64°C.

For each of the reactions, known malaria positive and negative samples from previously diagnosed patients served as controls. The following oligonucleotide primer sequences targeting the plasmodium spp. 18S RNA genes were used in this reaction;

Species	Base pair	Primer sequence
P. falciparum –	206bp	r FAL1- AACTGGTTTGGGAAAACCAAATATATT
		ACAATGAACTCAATCATGACTACCCGTC- r FA2
P. malariae –	144bp	r MAL- ATAACATAGTTGTACGTTAAGATAACCGC
		AAAATTCCCATGCATAAAAAATTATACAAA-r MAL2
P. ovale -	226bp	rOVA1-ATCTCTTTTGCTATTTTTTAGTATTGGAGA
		ATCAAGAATTTCACCTCTGACATCT- rPLU2

(iv) PCR Product Analysis

A 1.5 % Agarose gel in 1X TBE buffer (100ml 10X TBE+900ml Distilled water) was prepared and loaded with 12µl mixture of sample (from nest 2 reaction) and loading dye. A 100 base pair molecular marker was loaded to serve as a size marker for the malaria PCR products. This was run at a constant voltage of 90 volts for 1 hour using a Bio-Rad electrophoretic chamber. DNA was visualized under ultraviolet light (UV) illumination following staining with Ethidium Bromide. Images of the bands were taken using a Polaroid camera and the film was

processed according to the manufacturer's instructions. Gel documentation was done using aperture F8 and shutter speed B.

2.12 Data analysis

Data was entered in Microsoft excel where it was cleaned and later imported into SPSS version 11.0 software package for analysis. Univariate analysis was done in order to describe the distribution of single variables in the two groups. Tests of significance could not be performed on treatment outcomes because of the low numbers of patients in the case group.

2.13 Ethical and Management Consideration

Ethical clearance was sought from the University Of Zambia School Of Medicine Research and Ethics Committee. Permission to carry out the study was obtained from the Director of the University Teaching Hospital and the Ministry of Health for study sites outside Lusaka. Informed consent for participation in the study and HIV testing was obtained from parents or guardians of potential study subjects before enrollment. Each participant was given a unique study number for identification and all results were treated with utmost confidentiality.

2.14 Outcome Measures

The primary outcomes of the study were defined and categorized as: Adequate Clinical and Parasitological Response (ACPR), Early Treatment Failure (ETF), Late Clinical Failure (LCF), and Late Parasitological Failure (LPF). See Appendix B for detailed definitions.

CHAPTER FIVE: RESULTS

4.1 Baseline Characteristics in HIV –Malaria co-infection (Cases) and non HIV malaria infected Children (Controls)

The results in Table 1 show study participants characteristics at recruitment.

A total of 124 study participants were recruited into the study between December 2006 and July 2007. The study participants were recruited from 5 study sites namely: University Teaching Hospital, Chongwe district referral health centre, Chipata DHMT Clinic in Lusaka, Mpongwe Mission Hospital and Mpulungu district referral health centre. All the sites have unstable malaria transmission except for Mpulungu which has stable malaria transmission. Nine out of the 124 (7.3%) children recruited had HIV and malaria co-infection and made up the case group. One hundred and fifteen (92.7%) of the total recruited were HIV negative with malaria infection and made up the control group. Eighty (64.5%) of the 124 total recruited completed day 28 follow-up, 8 being cases and 72 as controls. Baseline characteristics of the 80 who completed day 28 follow-up are shown in Table 1. The mean age distribution among the cases and controls was 4.69 ±2.92 years. The mean age distribution was 5.31±3.77 and 4.62±2.83 for the cases and controls respectively. The difference in mean age distribution among the cases and controls was not statistically significant.

At enrollment, 50% of the children with HIV and malaria co-infection were more likely to present with complicated malaria than the non HIV malaria infected children. Children in both groups were treated with either Quinine or Coartem on day 0 upon recruitment and treatment was based on malaria severity. The total number of children treated with Coartem was 67 (83.8%) while those treated with Quinine was 13 (16.3%). Among the 67 children treated with Coartem, 3 (4.7%) were cases and 64 (88.9%) were controls. 37.5% and 62.5% of the children in the case group received Coartem and Quinine respectively. Of the 72 children in the control group, 64(88.9%) were treated with Coartem and 8 (11.1%) were treated with Quinine.

Table 1: **Baseline characteristics in HIV-malaria co-infected and non HIV malaria infected children who were recruited into the study**

Total Number Recruited		**HIV Malaria Group n=9**	**Non HIV malaria Group n =115**	**Total n=124**	
Baseline Characteristics	Completed Day 28 Follow-up	HIV Malaria Group n=8	Non HIV malaria Group n =72	Total n=80	Odds Ratio (95% CL)
Sex	Female	3 (37.5%)	43 (59.7%)	46 (57.5%)	0.405
	Male	5 (62.5%)	29 (40.3%)	34 (42.5%)	(0.09-1.83)
Age (yrs)	Mean ±SD	5.31±3.77	4.62±2.83	4.69±2.92	N/A
Disease severity	Complicated	4 (50.0%)	5 (6.9%)	9(11.3%)	13.40 (2-70.27)
	Uncomplicated	4 (50.0%)	67 (93.1%)	71 (88.8%)	
Treatment Drug	Coartem	3 (37.5%)	64 (88.9%)	67 (83.8%)	0.075
	Quinine	5(62.5%)	8(16.3%)	13 (16.3%)	(0.015-0.375)

4.2 Baseline clinical patient characteristics of the case and control groups

Baseline clinical patient characteristics are shown in Table 2. Children with HIV and malaria co-infection were 1.94 times more likely to present with pallor at recruitment as compared to the children in the malaria only group. One child out of the 8 children in the case group presented with convulsions while none of the children in the control group had this symptom. However, children in the control group were more likely to present with splenomegaly at recruitment than the HIV malaria co-infected children (22.2% and 12.5% respectively).

Table 2: **Patient clinical presentation at baseline among cases and controls**

Presenting symptoms and signs	HIV malaria Group n =8	Non HIV malaria Group n =72	Total n=80	Odds Ratio (95% CL)
Headache	4 (50.0%)	36 (50.0%)	40 (50.0%)	0.60 (0.10-3.20)
Cough	3 (37.5%)	38 (52.8%)	41 (51.3%)	0.89 (0.17-4.71)
Diarrhoea	4 (50.0%)	20 (27.8%)	24 (30.0%)	2.60 (0.48-14.0)
Convulsion	1 (12.5%)	0 (0.0%)	1 (1.3%)	N/A
Pallor	3 (37.5%)	17 (23.6%)	20 (25.0%)	1.94(0.32-10.84)
Respiratory Distress	1 (12.5%)	0 (0.0)	1 (1.3%)	N/A
Lymphadenopathy	2 (25.0%)	0 (0.0%)	2 (2.5%)	N/A
Splenomegaly	1 (12.5%)	16 (22.2%)	17 (21.3%)	0.50 (0.02-4.64)

4.3 Immunosuppression levels of the case and control groups

Table 3 shows the levels of immunosuppression in the HIV and malaria co-infected children and non HIV children infected with malaria. Immunosuppression was classified into three categories according to CD4 count levels; severe immunosuppression was defined as counts of CD4 <500 in the 1-5 year age group and <200 in the age group > 6 years. Moderate immunosuppression was defined as CD4 499-999 and 200-499 in the 1-5 year and > 6 year age groups respectively (Southern African HIV Clinicians Society, 2002).

Of the 48 children with no immunosuppression, 47 (97.9%) were controls and 1 (2.1%) was from the case group. A total of twenty six children had moderate immunosuppression with cases and controls having 12.5% and 34.7% respectively. Six (75%) out of 8 children in the case group had severe immunosuppression and only one moderate immunosuppressed child was recorded in this group. In the control group, 25 (34.7%) out of 72 children showed moderate immunosuppression but no severe immunosuppression was seen in this group. All the children with severe immunosuppression were HIV and malaria co-infected.

Table 3: **Immunological status of children in the case and control groups as determined by CD4 counts performed on EDTA blood by Facscount machine**

Immunosuppression Levels	Case Group n =8	Control Group n=72	Total
Normal	1 (12.5%)	47(65.3%)	48 (60.0%)
Moderate	1 (12.5%)	25(34.7%)	26 (32.5%)
Severe	6 (75.0%)	0 (0.0%)	6 (7.5%)
Total	8	72	80

Key: **No Immunosuppression- CD4 count >500 and >1000 cells/µl in children below 5 years and above 6 years respectively**
Moderate - CD4 counts of 499-999 and 200-499 in the 1-5 year and > 6 year age groups respectively
Severe - CD4 counts <500 in the 1-5 year age group and <200 in the age group > 6 years.

4.4 Malaria Parasitaemia levels in the case and control groups

Malaria parasite rates of children in the case and control groups are shown in Figure 1. The <1000 class represents the low parasitaemia level while 100,000 and >100,000 represents the moderate and high parasitaemia categories respectively.

The majority of the children in case and control groups had moderate parasitaemia counts in the range 1000-100,000 with the HIV and malaria co-infected group having slightly higher parasitaemia counts than the HIV negative malaria infected group in this category. In the high parasitaemia category, children in the control group were more likely to have higher parasitaemia than those in the case group.

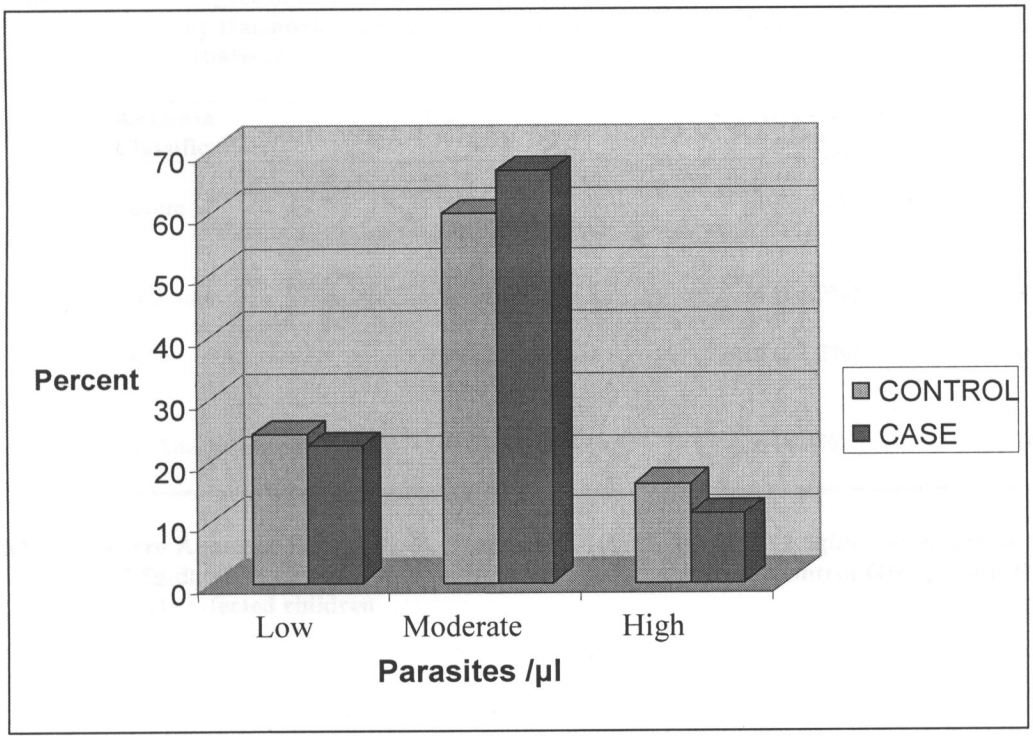

Figure 1: **Malaria Parasitaemia levels in cases and controls: Parasite densities were determined in thick Giemsa stained slides**

Key: **Low :< 1000 parasites/µl, Moderate: 1000-100 000 parasites/µl, High >100000 parasites/µl**

4.5 Levels of anaemia in the case and control Groups

The levels of anaemia in HIV-malaria co-infected children (cases) and non-HIV malaria infected children (controls) is shown in Table 4. The levels of anaemia were determined and classified into 4 categories according to haemoglobin levels. Among the 8 children in the case group, 4 (50%) presented with severe anaemia while moderate and mild anaemia were found in 3(37.5%) and 1 (12.5%) of the children respectively. No normal haemoglobin levels were recorded in this group. In the control group however, the majority (63.9%) of the children presented with moderate anaemia with only 4 (5.6%) children having severe anaemia. Six (8.3%) of the children in this group had normal haemoglobin.

Table 4: **Degree of anaemia in the case and control groups as determined by Haemoglobin levels performed using an ABX Micros Analyser**

Anaemia Classification	Case Group n=8	Control Group n=72	Total
Severe	4(50.0%)	4 (5.6%)	8 (11.3%)
Moderate	3 (37.5%)	46 (63.9%)	49 (61.3%)
Mild	1 (12.5%)	16 (22.2%)	16 (20.0%)
No Anaemia	0 (0.0%)	6 (8.3%)	6 (7.5%)

KEY: **Severe Anaemia Hb < 5.0g/dl, Moderate 6-10g/dl, Mild 11-12.5g/dl, No Anaemia Hb >12.5g/dl. Case Group: HIV- malaria co-infected children; Control Group: Non HIV malaria infected children**

4.6 Disease morbidity in HIV-Malaria co-infected and non HIV malaria infected children

The table below shows disease morbidity in HIV-Malaria co-infected and non HIV malaria infected children. The geometric mean for malaria parasitaemia for the HIV and malaria co-infected children and non HIV malaria infected children was 11501.32 and 7550.83 respectively. Though the geometric mean parasitaemia was higher in the case group than the control group, this difference was not considered to be statistically significant (p-value =0.841). The mean haemoglobin in the case group was lower (6.62 ±2.4) than that in the control group (9.28 ±2.6). The difference was statistically significant with a p-value of 0.003. Differences in mean white blood counts in both groups were not significantly different (8.51±6.05 and 7.40±3.20; p-value 0.36). However, mean CD4 count differences in the two groups were statistically significant (356±142.99 and 1032.50±436.64 respectively, p-value <0.001). The mean CD4 count was lower among the children in the case group.

Table 5: Disease Morbidity in HIV-Malaria co-infected and non HIV malaria infected children

Morbidity		Case Group n =8	Control Group n =72	Total n= 80	p-value
Temperature (°C)	Mean ±SD	37.85±1.090	37.72±1.175	37.73±1.1605	
Parasite Density/ul	Range	440-253840	48-812000	48-812000	
	Geo-Mean	11501.32	7550.83	±	0.841
Haemoglobin (g/dl)	Mean ±SD	6.62 ±2.71	9.55±2.40	9.23±2.603	0.003
White Blood Cell	Mean ±SD	8.51±6.05	7.40±3.20	±	0.360
CD4 Count/µl	Range	270-690	237-2000 1171.86(±445.1	237-2000	
	(Mean ±SD)	(356±138.47)	8)	1093.46±485.626	<0.001

Case Group: HIV- malaria co-infected children; Control Group: Non HIV malaria infected children

4.7 Malaria treatment outcomes according to HIV -1 Serostatus

Table 6 shows malaria treatment outcomes in HIV and malaria co- infected children and non HIV malaria infected children. The number of children followed-up to day 28 in both groups was 80. Among the 64 (80%) Adequate Clinical and Parasitological Response (ACPR) recorded, 7 (10.9%) were in HIV and malaria co-infected children and 57 (89.1%) were in non HIV malaria infected children. A total of 16 (20%) treatment failures (TF) were recorded of which 15 (93.8%) were in the control group while one (6.2%) treatment failure was recorded in the case group. ACPR and TF within the case group was 87.5% and 12.5% respectively. In the control group, ACPR and TF were 57 (79.2%) and 15 (20.8%) respectively. Children in the case group appear to have responded better to antimalarial treatment.

Table 6: **Malaria Treatment Outcome according to HIV -1 Serostatus**

Outcome	HIV- Malaria Group n =8	Non HIV Malaria Group n =72	Total
ACPR	7 (87.5%)	57 (79.2%)	64
Total TF	1 (12.5%)	15 (20.8%)	16
Total	**8 (100%)**	**72 (100%)**	**80**

ACPR; Adequate Clinical and Parasitological Response, TF; Treatment Failure (see Appendix B for definitions) based on microscopy and PCR.

4.8 Treatment outcomes in the case and control Groups in relation to specific antimalarial drug

Antimalarial treatment outcomes in HIV- malaria co-infected and non HIV malaria infected children are shown in Table 7. Based on the WHO classification, (WHO, 2003) treatment outcomes were categorised into four; Adequate Clinical and Parasitological Response (ACPR), Early Treatment Failure (ETF), Late Treatment Failure (LTF) and Late Parasitological Failure (LPF) see Appendix B for definitions). Treatment outcome was based on positive slide and positive PCR for malaria. Of the 16 treatment failures, 4 were positive by microscopy and 14 were positive by PCR. Two antimalarial drugs namely: Coartem and Quinine were given to children upon recruitment based on a positive blood slide. Treatment outcome was monitored for a period of 28 days according to WHO guidelines (WHO, 2003).

Table 7: Treatment outcomes based on antimalarial drug in the cases and controls

Treatment Drug	Treatment Outcome	Case group n= 8	Control Group n=72	Total
Coartem	ACPR	2 (66.7%)	50 (78.1%)	52 (80%)
	LPF	1(33.3%)	14 (21.9%)	15(22.4%)
	Total	3	64	67
Quinine	ACPR	5(100.0%)	7 (87.5%)	12 (92.3%)
	LTF	0 (0.0%)	1 (12.5%)	1 (7.7%)
	Total	5	8	13
Total		8	72	80

ACPR- Adequate Clinical and Parasitological Response, LPF-Late Parasitological Failure, LTF-Late Treatment Failure

A total of 64 children were given Coartem of which 3 were cases and 64 were controls. ACPR was recorded in 52 (80%) children (2 cases and 50 controls). Fifteen treatment failures were recorded in this treatment group; 14 were among controls and 1 was in the case group. Within the case group, 2 (66.7%) out of the 3 children responded adequately while 1 (33.3%) developed LPF by day 14. In the control group, ACPR and LPF was 50 (78.1%) and 14 (21.9%) respectively.

Thirteen out of the 80 children followed- up to day 28 were treated with Quinine. Among these 13 children in this treatment group, 12 (92.3%) had ACPR while 1 (7.7%) LTF was recorded. However within the case group, all the 5 (100%) children treated with Quinine had ACPR by day 28. The treatment failures seen in this treatment group were among children in the control group. No early treatment failures were recorded in children treated with either Quinine or Coartem in the case and control groups. ACPR for children in the control group treated with Quinine was 7 (87.5%).

4.9 Malaria treatment outcomes in relation to malarial disease severity in the case and control groups

Treatment outcomes in relation to disease severity in the HIV-malaria co-infected and non HIV malaria infected children are shown in table 8. Out of the 80 children who were followed-up to day 28, 9 presented with complicated malaria while 71 had uncomplicated malaria. Among the 9 complicated cases, 4 were children with HIV-malaria co-infected and 5 were HIV malaria infected children. ACPR was recorded in all the 9 (100%) children in both the case and control groups. No treatment failures were seen in complicated malaria cases. However, poor response to antimalarial treatment was noted in uncomplicated cases with 20.9% and 25% LPF occurring in the control and case groups respectively. Only one late treatment failure was recorded in the control group and none was recorded in the case group.

Table 8: **Treatment outcome in complicated and uncomplicated malaria cases in the case and control groups**

Disease severity	Treatment Outcome	Case Group n= 8	Control Group n=72	Total
Complicated	ACPR	4 (100.0%)	5 (100.0%)	9 (100.0%)
	LPF	0 (0.0%)	0 (0.0%)	0 (0.0%)
	LTF	0 (0.0%)	0 (0.0%)	0 (0.0%)
	Total	4	5	9
Uncomplicated	ACPR	3 (75.0%)	52 (77.6%)	55 (77.5%)
	LPF	1 (25.0%)	14 (20.9%)	15 (21.1%)
	LTF	0 (0.0%)	1 (1.5%)	1 (1.4%)
	Total	4	67	71
Total		**8**	**72**	**80**

Case Group: **HIV- malaria co-infected children;**
Control Group: **Non HIV malaria infected children**
Complicated malaria: Haemoglobin <5.0g/dl; Uncomplicated: Haemoglobin >5.0g/dl

4.10 Treatment outcomes in the case and control groups according to age Group

According to Table 9, all the children in the 2-5 years age group with HIV malaria co infection had a 100% adequate response to antimalarials. Forty (74.1%) of the 72 non HIV malaria infected children in this age group had ACPR while 13 (24.1%) and 1(1.9%) developed LPF and LTF respectively. Among the 19 children above the age of 6 years who responded well to antimalarials, 2 were in the case group while 17 were from the control group.

In children above 6 years of age, ACPR and LPF within the case group was 66.7% and 33.3% respectively. Treatment outcome within the control group was 94.4% and 5.6% for ACPR and LPF respectively. No LTFs in this age group were recorded.

Table 9: Treatment outcomes as stratified by age group

AGE GROUP	Treatment Outcome	Case Group n=8	Control Group n=72	Total
2- 5 YRS	ACPR	5 (100%)	40 (74.1%)	45 (76.3%)
	LPF	0 (0.0%)	13 (24.1%)	13 (22.0%)
	LTF	0 (0.0%)	1 (1.9%)	1 (1.7%)
	Total	5	54	59
> 6 YRS	ACPR	2 (66.7%)	17(94.4%)	19 (90.0%)
	LPF	1 (33.3%)	1 (5.6%)	2 (10.0%)
	LTF	0 (0.0%)	0 (0.0%)	0 (0.0%)
	Total	3	18	21
Total		**8**	**72**	**80**

Case Group: HIV- malaria co-infected children; Control Group: Non HIV malaria infected children

4.11 Treatment outcomes in relation to degree of immunosuppression

Treatment outcomes based on immunosuppression levels is shown in Table 10. All the 6 (100%) children with severe immunosuppression were HIV malaria co-infected and had ACPR by day 28. LPF was recorded in one child with moderate immunosuppression. In the control group, LPF was 5 (20.0%) and 9 (19.1%) in children with moderate and normal immunity respectively. Only one child with moderate immunosuppression developed LTF in the control group.

Table 10: Treatment outcomes in relation to levels of Immunesuppression in Children

Immunosuppression	Treatment Outcome	Case group n= 8	Control Group n=72	Total
Normal	ACPR	1 (100.0%)	37 (78.7%)	38 (79.2%)
	LPF	0 (0.00%)	9 (19.1%)	9 (18.8%)
	LTF	0 (0.00%)	1 (2.1%)	1 (2.1%)
	Total	1	47	48
Moderate	ACPR	0 (0.00%)	20 (80.0%)	20 (76.9%)
	LPF	1 (100.0%)	5 (20.0%)	6 (23.1%)
	Total	1	25	26
Severe	ACPR	6 (100.0%)	0 (0.0%)	6 (100.0%)
	Total	6		6
Total		**8**	**72**	**80**

Severe immunosuppression: **CD4 <500 in the 1-5 year age group and <200 in the age group > 6yrs**

Moderate immunosuppression: **CD4 499-999 in 1-5 yrs and 200-499 > 6yrs**

No Immunosuppression: **CD4 count >500 and >1000 cells/µl in children below 5 years and above 6 years respectively (Southern African HIV Clinicians Society, 2002)**

4.12 Re-Infections and Recrudescent among the case and control groups

Sixteen of the 80 children followed-up to day 28 had post treatment parasites on day 14 and 21 respectively. Four of the 16 samples were positive by microscopy and 12 by both microscopy and PCR. In order to distinguish re-infection parasites from recrudescent, all the 16 positive post treatment samples were genotyped. Out of the 16 post treatment malaria positive samples recorded, 5 (31.3%) were due to re-infections and 11 (68.7%) were recrudescents. All the 4 re-infections were among children in the control group. Only recrudescence and no re-infections were observed among the HIV-Malaria co-infected children (Cases). Ten of the 16 recrudescent parasites were seen in 6.2% and 62.5% of the cases and controls respectively. After genotyping post treatment positive malaria samples, 11 treatment failures were seen among the 80 recruited patients. Treatment failures within the case and control groups were 1 (12.5%) and 10 (13.8%) respectively.

Table 12: Incidence of Re-infections and Recrudescences in HIV –malaria co-infected and Non HIV malaria infected children

Study Groups	Re-infection	Recrudescence	Total
Non-HIV malaria Infected Children	5/16 (31.3%)	10/16 (62.5%)	15/16 (93.8%)
HIV malaria Infected Children	0 (0.0%)	1/16 (6.2%)	1/16 (6.2%)
Total	**5/16 (31.3%)**	**11/16 (68.7%)**	**16/16 (100%)**

CHAPTER FIVE: RESULTS

5.1 Study Discussion

This study was conducted in order to better understand the effect that HIV infection may have on antimalarial treatment outcome in children. This was based on the assumption that HIV 1 infection is a risk factor for severe malaria and antimalarial treatment failures in children.

As malaria susceptibility and response to antimalarial treatment is dependent on both the humoral and cellular immunity, any alteration in the host's immune defence mechanisms against many pathogens malaria inclusive may result in reduced response against such diseases and might be associated with high malaria parasitaemia and severe disease (Whitworth, 2006). In endemic areas, immunity to malaria infection develops after repeated exposure to parasites and therefore children below the age of 5 years are at high risk of developing malaria infections. Cell Mediated and humoral immunity are responsible for controlling malaria infections (Perlmann *et al*, 2002). However, in HIV infections with decreased immunity, this mechanism is impaired and may result in increased parasitaemia and clinical illness with reduced antimalarial treatment response (Whitworth *et al*, 2000).

Our baseline data has shown that children with HIV and malaria co-infection were more likely to present with severe malaria than those without HIV infection. The majority of the children with complicated malaria came from Lusaka, an area of unstable malaria transmission. These findings are consistent with results from a study conducted by Chirenda *et al* (2000) which demonstrated that people with HIV infection living in areas of unstable malaria transmission were at higher risk of developing complicated malaria than those in areas of high or stable malaria transmission. Their study also confirms results by Grimwade *et a*l which show that children with HIV infection co- infected with malaria in Kwazulu Natal, an area of unstable malaria transmission were more likely to experience severe disease, coma

and even death (Grimwade *et al*, 2003). The malaria presentations in such areas are similar in all age groups because immunity to the disease is limited.

Malaria mean parasitaemia levels were slightly higher among HIV and malaria co-infected children than in the HIV negative malaria infected children though this difference was not statistically significant. This finding is in agreement with a study in Uganda that has documented similar findings with regard to lower parasitaemia levels among non pregnant adults co-infected with HIV and malaria (Kamya *et al* 2005). Similarly, Villamor *et al* (2003) also showed a negative association between HIV infection and malaria parasitaemia and low malaria prevalence among HIV–malaria co-infected children.Whitworth on the other hand had demonstrated higher and significant parasitaemia levels among adult individuals living with HIV malaria co-infection having low CD 4 counts (Whitworth *et al,* 2000). The lack of consistency in these three studies and others on parasitaemia levels could be attributed to the fact that the study populations were different in age.

In this study, low haemoglobin levels and severe anaemia were observed in children infected with HIV presenting with malaria. These findings have also been documented by Shah *et al* (2006) who showed that anaemia in the presence of decreasing CD4 levels was a risk factor for treatment failures in people infected with HIV and malaria. However, in our study we did not find any association between anaemia and treatment failure among children in this group.

The study showed that children infected with HIV and having immunosuppression (270- 690 for cases and 237-2000 for controls) as classified by CD4 counts were less likely to develop treatment failures. The majority of the children in the case group had severe immunosuppression and a few children in the control group presented with moderate immunosuppression. Severe or moderate immunosuppression in both groups was not a factor for treatment failure. This is in contrast to reports by other studies (Shah et al 2006). Shah *et al* (2006) demonstrated that SP efficacy is compromised in HIV and malaria co-infected adults. The reduction in the treatment response from their study could have been due to resistance by the malaria parasites towards SP. Our study findings are similar

to recent findings by Lauer *et al* (2007) that have documented the lack of correlation between decreasing immunological response and antimalarial treatment failures among HIV infected individuals.

Non HIV malaria infected children (control Group) on the other hand had slightly higher treatment failures than the HIV and malaria co-infected children. The reason for this difference could be attributed to the fact that as most children in the case group presented with complicated malaria of anaemia, they were more likely to be treated with efficacious drugs such as Quinine with close monitoring in hospital for a number of days. On the other hand, the majority of the children in the control group had uncomplicated malaria and were therefore treated with Artemether Lumenfantrine (Coartem) as outpatients. Monitoring of compliance to the drug uptake by the children was not possible as it was beyond the study's capabilities.

After stratification of children into age groups, the study found that children below the age of 5 years were more likely to develop treatment failures than children above the age of 5 years. Similar findings have been shown in a study by Dorsey *et al* (2004) who concluded that decreasing age was a risk factor for treatment failure since immunity to malaria infections is developed over a number of years through repeated exposure to malaria parasites. Therefore, children in this age group are more likely to suffer severe malaria as they may not have developed an immune system strong enough to contain the parasites within their bodies.

5.2 Study Limitations

It was difficult to reach the calculated sample size of 105 HIV and malaria co-infected children due to the low prevalence of HIV-Malaria co-infected children. The programs embarked by the Zambian government over the years to scale up malaria control such as: change in treatment policy, increase in the use of insecticide treated nets and indoor residue spraying, have resulted in a significant reduction in the numbers of malaria cases. Indeed several studies have incidentally reported this similar observation (Kamya *et al,* 2006). In order to increase the

sample probability space more study sites were added to the initial sites and the study was prolonged beyond the planned time frame.

Most tests of significance could not be applied to test the hypothesis because the study did not assume randomization and equal size for both the control and the cases.

5.3 Conclusion

This study shows that children with HIV infection co-infected with malaria were more likely to present with severe malaria and anaemia than HIV negative malaria infected children. Malaria mean parasitaemia levels were slightly higher among children in the case group than children in the control group.

Though the study findings have shown that the risk of developing treatment failure is less likely among children with severe immunosuppression, the results may not be significant and conclusive due to a number of factors aforementioned.

5.4 Recommendations

In view of these study observation we recommend that future research with adequate study periods and better randomized study groups are imperative to better address and elucidate the impact of HIV infection on antimalarial treatment outcomes in children.

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Appendix A: Procedures and Follow-Up

PROCEDURE	DAY 0	DAY 1	DAY 2	DAY 3	DAY 7	DAY 14	DAY 21	DAY 28	Any other day
Clinical Assessment	X	X	X	X	X	X	X	X	X
Blood slide-Malaria Examination/ Parasitaemia	X		X	X	X	X	X	X	X
Haemoglobin/ FBC	X								
HIV Test	X								
CD 4 COUNT	X								
Blood for PCR	X					X			
Treatment	X	X	X	X	X				

Day 0:

Clinical assessment - referral in case of severe malaria/danger signs

Measurement of axillary temperature

Parasitological assessment

Informed consent - enrolment

Measurement of weight (and height) - treatment, first dose

Haemoglobin/haematocrit, blood sampling for PCR

Day 1:

Clinical assessment - referral in case of severe malaria/danger signs

Measurement of axillary temperature

Parasitological assessment in case of severe malaria/danger signs

Treatment, second dose or alternative treatment in case of early treatment failure

Day 2:

Clinical assessment - referral in case of severe malaria/danger signs

Measurement of axillary temperature

Parasitological assessment

Treatment, third dose or alternative treatment in case of early treatment failure

Day 3, Day 7, Day 14, Day 21 and Day 28:

Clinical assessment - referral in case of severe malaria/danger signs

Measurement of axillary temperature

Parasitological assessment

Alternative treatment in case of treatment failure

Optional: Haemoglobin/haematocrit (Day 14, Day 28), blood sampling for PCR (Any other day on or after Day 14 in case of failure)

Any other day:

Clinical assessment - referral in case of severe malaria/danger signs

Measurement of axillary temperature

Parasitological assessment

Alternative treatment in case of treatment failure

Appendix B

Classification of Treatment Failure in Areas of Low to Moderate Transmission

Therapeutic response are classified into three categories namely; early treatment failure (ETF), late clinical failure (LCF), Late parasitological failure (LPF) and adequate clinical and parasitological response (ACPR).

ETF -Development of danger signs or severe malaria on Day 1, Day 2 or Day 3, in the presence of parasitaemia

-Axillary temperature _>37.5 degrees Celsius on Day 2 with parasitaemia >of Day 0.

-Axillary temperature _>37.5 degrees Celsius on Day 3 in the presence of parasitaemia

-Parasitaemia on Day 3 _> 25 % of count on Day 0.

LTF -Development of danger signs or severe malaria in the presence of Parasitaemia on any day from Day 4 to Day 14, without previously meeting any of the criteria of early treatment failure;

-Axillary temperature _> 37.5 degrees Celsius in the presence of parasitaemia on any day from Day 4 to Day 14, without meeting any of the criteria of early treatment.

LPF -Presence of parasitaemia on any day from Day 7 to Day 28 and axillary

Temperature < 37.5, without previously meeting any of the criteria of Early Treatment Failure of Late Treatment Failure

ACR -Absence of parasitaemia on day 14 irrespective of axillary temperature, without previously meeting any of the criteria of early or late treatment failure;

-Axillary temperature < 37.5 irrespective of the presence of parasitaemia, without previously meeting any of the criteria of early or late treatment failure.

Appendix C: Immunological categories for children with HIV infection

Age of children

	>12months		1-5 years		6 12 years	
Immunological Category	CD4/ml	CD4%	CD4/ml	CD4%	CD4/ml	CD4%
No immunosuppression	>1 500	≥25	≥1 000	≥25	≥500	≥25
Moderate immunosuppression	750-1 499	15-24	500-999	15-24	200-499	15-24
Severe immunosuppression	<750	<15	<500	<15	<200	<15

Classification according to Southern African HIV Clinicians Society, 2002

Appendix D: WHO Malaria Classification

Severe manifestations and complications of *P. falciparum* malaria
In a patient with falciparum malaria in whom other diseases have been excluded, the presence of one or more of the following manifestations is sufficient for a diagnosis of severe falciparum malaria.

Malaria Case Classification	Definition
1. Cerebral malaria	Unarousable coma (Blantyre Coma scale- Score Out of 5 Stages 1,2,3, or 4
2. Severe Anaemia	Hemoglobin < 3.1 mmol/l or 5g/dl
3. Metabolic acidosis	Blood pH of <7.35 or plasma bicarbonate concentration of < 22 mmol/L
4. Renal failure	Urine output of < 400 ml in 24 hrs or < 12ml/kg per 24 hrs
5. Pulmonary Oedema	Breathlessness, bilateral crackles
6. Hypoglycaemia	Blood glucose concentration of less than 2.2 mmol/l or < 40 mg/dl)
7. Haemoglobinuria	Black water fever
8. Circulatory Collapse	Systolic BP < 50 mmHg in children 1-5 years or <70 in older ones
9. Hyperpyrexia	Temperature above 40^0C
10. Convulsions	> 2 seizures in 24 hours with regaining of consciousness
11.Disseminated Intravascular Coagulation	Bleeding and clotting disturbances

Appendix E

Information Sheet

I, **Mable Mwale Mutengo** student at the University of Zambia, School of Medicine, will be conducting a research study on the effect of HIV on anti malaria treatment in both HIV -positive patients with malaria and HIV -negative patients with malaria.

Procedure:

In order to achieve this goal a small amount of blood, about 2 teaspoons, will be collected from your child to see if your child has malaria and HIV. Your child will be treated on day zero if she or he has malaria and will be followed up for 28 days.

The treatment drug is either **Coartem or Quinine.**

If you agree to have your child participate in this study, we would like you to come to the clinic 6 more times over the next four weeks so that we monitor your child's progress. At each of these visits your child will undergo clinical examination and on three of these visits a small amount of blood will be taken to check if your child still has malaria.

Benefits:

Your participation is completely voluntary and it will not cost you or your family anything. All costs relating to transport to and from the hospital will be covered by the study.

You may withdraw your child from the study at anytime and this will not affect your child's standard health care. Your child will benefit from this study in that he or she will be monitored for the next 28 days. If your child continues to suffer from malaria or has HIV, he or she will be referred for further clinical management.

Risks and Discomforts:

The study is very safe and will not cause any physical harm to your child. The child may feel a little discomfort when blood is being drawn.

The emotional and psychological effect of an HIV test result will be dealt with by the counselor during pre and post HIV counseling.

All information given and results shall be treated with utmost confidentiality.

PARTICIPANT'S CONSENT FORM

I ---------- read the information on the purpose of the study/ it has been explained to me.

I do understand that this study will improve malaria case management in HIV-Malaria co-infected children.

I also understand that all results and information I give shall be treated with strict confidentiality and that I have a choice to have my child participate or withdrawn from the study at any time and that my child's withdrawal will not affect his/her standard care and treatment.

I therefore, accept that my child/dependent participates in the study with the above understanding.

I also **choose / choose not** to know the HIV results of my child (Please circle the appropriate phrase.)

Name of child_____

*Name of parent/Guardian_____Signature/_____

*Delete appropriate Thumb print

Name of investigator_____Signature_____

Witness

_____Signature_____

Date _____

Contact Person

Mable M Mutengo
University of Zambia
School of Medicine
Lusaka
Tel: 097-7- 842352

OR

The Research Ethics Committee
P.O Box 50110
Lusaka.
Tel:250067

HIV AND MALARIA CO-INFECTION: EFFECT OF HIV ON ANTIMALARIAL TREATMENT OUTCOMES IN CHILDREN (CLINICAL DATA COLLECTION SHEET)

STUDY SITE	Health facility's name						Town:					District/Province		
PATIENT	Identity number						Age/(years)		Sex (M/F)			Weight (kg)		Height (cm):
PRESCRIBED ANTIMALARIA	Drug name						Contact home address:							
			Dosage											
DAY	0	1	2	3	4	5	6	7		9	10	11	12	13	14
Date															
History of fever last 24[hrs] (Y/N)															
Axillary temperature (^{o}c)															
Headache Y/N															
Diarrhoea (Y/N)															
Cough (Y/N)															
Convulsions (Y/N)															
Pallor (Y/N)															
Jaundice(Y/N)															
Respiratory distress (Y/N)															
Generalised lyphadenopathy(Y/N)															
Coma *															
Splenomegaly (Y/N)															
Any side effects of antimalarials*															
Antimalarial Tx last 2 wks (Y/N)*															

STUDY SITE	**Health facility's name**			**Town:**						**District/Province**			
PATIENT	Identity number									Drug name			
DAY	15	16	17	18	19	21	22	23	24	25	26	27	28	29
Date														
Danger sign (Y/N)														
History of fever last 24^{hrs}(Y/N)														
Axillary temperature (^{o}c)														
Headache(Y/N)														
Diarrhoea Y/N)														
Cough ((Y/N)														
Convulsions Y/N)														
Pallor (Y/N)														
Jaundice (Y/N)														
Respiratory distress (Y/N)														
Generalised lyphadenopathy (Y/N)														
Coma*														
Splenomegaly (Y/N)														
Provisional Diagnosis*														
Any side effects of antimalarials*														
RVD stage*														

Overall assessment: Early treatment Failure (ETF) Late clinical Failure (LCF) Late parasitological Failure (LPF) Adequate clinical and Parasitological Withdrawn (WTH)

*Provisional Diagnosis:

HIV/ MALARIA CO-INFECTION IN CHILDREN: PATIENT LABORATORY DATA FORM B (To be filled in by Study Investigator and Laboratory Technologist)

Study ID Number	DD MM YY	Investigator: Technologist:

1.1 Patient Hospital File:.....

1.2 Date and Time Sample Collected: Date.....Time.....

1.2 Date and Time Sample Received: Date.....Time.....

HAEMATOLOGY (FBC AND DIFFERENTIALS)

3.0 WBC 3.0 HB 3.1 HCT 3.2 RBC? 3.3 PLT? 3.4 PCT

3.5 MCV? 3.6 MCH? 3.7 MCHC? 3.8 RDW? 3.10 MPV?

3.9 PDW?

PARASITOLOGY-

4.1 Parasitaemia (Parasites/μL)? 4.1 Infective Plasmodium? 4.1a P. falciparum?

4.1 b P.Malariae? 4.1 c P. ovale? 4.1 d P. vivax?

PARASITOLOGY-PCR RESULTS

5.0 Recrudescence 5.1 Re- infection

IMMUNOLOGICAL PROFIILES

7.1 Total WBC? 7.2 Lymphocyets%? 7.3 Monocytes%?

7.4 Granulocytes%? 7.5 Basophils? 7.6 Eosinophils

8.1 CD4 counts? 8.2 CD8 Counts? 8.3 CD4/8 Ratio?

9.0 RVD Status? 9.1 RVD Immunogical Staging?

Follow-up blood slide Results

Day 1 Day 3 Day 7 Day 14 Day 21 Day 28

THE UNIVERSITY OF ZAMBIA

RESEARCH ETHICS COMMITTEE

Telephone: 260-1-256067
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Ridgeway Campus
P.O. Box 50110
Lusaka, Zambia

Assurance No. FWA00000338
IRB00001131 of IORG0000774

15 August, 2006
Ref.: 017-06-06

Ms Mable Mwale Mutengo
Department of Biomedical Sciences
School of Medicine
University of Zambia
P.O. Box 50110
LUSAKA

Dear Ms Mutengo,

RE: RESEARCH PROPOSAL ENTITLED: **"MALARIA AND HIV CO-INFECTION: EFFECT OF HIV INFECTION ON ANTIMALARIAL TREATMENT OUTCOMES IN CHILDREN"**

The above research proposal was presented to the Research Ethics Committee meeting on 5 July, 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.

Yours sincerely,

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

Date of approval: 15 August, 2006 **Date of expiry:** 14 August, 2007

THE UNIVERSITY OF ZAMBIA

RESEARCH ETHICS COMMITTEE

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Ridgeway Campus
P.O. Box 50110
Lusaka, Zambia

14 March, 2007
Our Ref: 017-03-06

Ms Mable Mutengo
Department of Biomedical Sciences
School of Medicine
University of Zambia
LUSAKA

Dear Ms Mutengo,

RE: ADDITIONAL STUDY SITES: **"MALARIA AND HIV CO-INFECTION: EFFECT OF HIV INFECTION ON ANTIMALARIAL TREATMENT OUTCOMES IN CHILDREN"**

We acknowledge receipt of your letter dated 26 February, 2007.

We are pleased to inform you that the addition of Chipata, Mpongwe, Mpulungu, Kitwe and Solwezi Districts as well as Lusaka DHMT clinics to the study sites is approved. We also take note that the addition of study sites has been necessitated by the low numbers of malaria cases in the initial sites.

With best wishes.

Yours sincerely,

Dr E. Munalula-Nkandu
SECRETARY

All Correspondence should be addressed to the
Permanent Secretary
Telephone: +260 1 253040/5
Fax: +260 1 253344

In reply please quote:

No.....

REPUBLIC OF ZAMBIA

MINISTRY OF HEALTH

NDEKE HOUSE
P. O. BOX 30205
LUSAKA

2nd March, 2007

The Dean – School of Medicine
University of Zambia
LUSAKA

Dear Dr. Shinondo,

RE. AUTHORITY TO CONDUCT MALARIA FIELD STUDY; MASTER OF SCIENCE MEDICAL PARASITOLOGY STUDENTS – UNZA.

We acknowledge receipt of your letter on the above subject.

The Ministry of Health has no objection to your request. You may therefore go a head and conduct your research study. However, the following guidelines need to be adhered to;

1. Consent from patients / clients obtained and ethical consideration taken into account.
2. Picture / reports need to be cleared and seen by Ministry of health prior to use outside the country.
3. Students need to report to Mpulungu and Mpongwe District Directors of Health respectively and work closely with them.
4. Also ensure that all required formalities with the Ethics Committee are followed.

Dr. Victor M. Mukonka
DIRECTOR OF PUBLIC HEALTH AND RESEARCH

Cc. The District Director of Health – Mpulungu
The District Director of Health - Mpongwe

P.O. Box 50827
Lusaka
Tel: +260-1-235554
Fax: +260-1-236429

In reply please quote
No.....

Republic of Zambia

MINISTRY OF HEALTH
LUSAKA DISTRICT HEALTH MANAGEMENT TEAM

21st February,2007

The Course coordinator
Medical Parasitology
University of Zambia
Ridgeway Campus
LUSAKA.

Dear Sir,

RE: RESEARCH / DATA COLLECTION

Be informed that permission has been granted for your students to carry out the above planned activities in our health facilities.

However, this should be done with minimal disruption to the day to day activities at the Health Centres

By copy of this letter Health Centre In- Charges are informed forthwith.

Your usual cooperation will be highly appreciated.

Yours faithfully,

DR. M. KABASO
CLINICAL CARE MANAGER
FOR/DISTRICT DIRECTOR OF HEALTH

C.C: Health Centre In- Charges
C.C: Mrs. Mutembo and Mrs Sitali

7th December 2006

Dr C J Shinondo,
University of Zambia,
School of Medicine,
P.O. Box 50110,
LUSAKA.

Dear Dr Shinondo,

RE: PERMISSION FOR LUNGOWE SITALI AND MABLE MUTENGO, MSc MEDICAL PARASITOLOGY STUDENTS TO CONDUCT RESEARCH IN THE DEPARTMENT OF PAEDIATRICS

We are in receipt of your letter dated 11th September 2006, on the above-mentioned subject.

We are happy to inform you that permission for the two students to conduct the research in the Department of Paediatrics, has been granted. This is on condition that half of the budget for miscellaneous costs be paid to University Teaching Hospital to take care of the utility and disposal of waste bills.

Yours Sincerely,
UNIVERSITY TEACHING HOSPITAL

Dr T Kafula
Deputy Managing Director
For/MANAGING DIRECTOR

Cc Managing Director
Cc Ms Lungowe Sitali - Student
Cc Mrs Mable Mutengo - Student