INFLUENCE OF HAPTOGLOBIN GENOTYPES ON SUSCEPTIBILITY TO MALARIA AND THE EFFECT OF MALARIA PARASITEMIA TO THE HAPTOGLOBIN LEVELS IN CHILDREN

BY

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ABSTRACT

Haptoglobin gene encodes for an acute phase protein; Haptoglobin and is expressed in three different polymorphic forms, Hp^{1-1}, Hp^{1-2} and Hp^{2-2}. Various studies on pathogenesis of Malaria maintain that Haptoglobin polymorphism influences host susceptibility to *Plasmodium falciparum* infection. A number of studies provide evidence that, individuals carrying Hp^{2-2} genotype are resistant to both Malaria infection and development of severe disease although some have shown that no influence manifested by the genotype. The present study aims on drawing clear information on the effect of the individual host Haptoglobin genotype to Malaria, focusing on the more Malaria susceptible group; Children under five years of age. The study also investigated how Plasma Haptoglobin level in the three Haptoglobin genotypes is affected by Malaria infection. A cohort of 344 under five years of age children in Morogoro was followed for an average of one year, where each child attended clinic once in every four weeks. Haptoglobin genotyping was done by PCR while was done by indirect ELISA. Among the 344 children, the proportion of the three genotypes (Hp^{1-1}; $Hp1^{-2;}$ Hp^{2-2}) was 85; 171; 88 and this obeys the Hardy-Weinberg equilibrium. Microscopic examination of blood smears found that positive cases for Malaria were 11.7%, 11.6% and 12.4% for the genotypes Hp^{1-1}, Hp^{1-2} and Hp^{2-2} respectively. Haptoglobin levels were significantly higher with negative parasitemia when compared with Hp^{1-1} ($p=0.01$). The variation in the level of plasma Haptoglobin level due to Malaria infection was significantly higher in Hp2-2 when compared to Hp1-1 when compared by unpaired t-test ($p=0.03$). This study concluded that at the age below five years the Haptoglobin genetic polymorphism neither influences susceptibility to Malaria infection nor the disease development after infection and the magnitude of drop in plasma Haptoglobin level due to Malaria infection is higher in Hp^{2-2} than in Hp^{1-1} individuals.

DECLARATION

I, Benigni Alfred do hereby declare to the senate of Sokoine University of Agriculture that this Dissertation is my own work and effort and that it has not been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged.

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The above declaration is confirmed by

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LIST OF ABBREVIATIONS AND SYMBOLS

BS+	Blood slide with positive parasitemia
BS-	Blood slide with negative parasitemia
BSA	Bovine Serun Albumin
CD163	Cluster of Differentiation 163
CO	Carbon Monoxide
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythiamidine triphosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EIR	Entomological Inoculation Rate
ELISA	Enzyme Linked Immunosorbent Assay
g	gravity
G6PD	Glucose-6-phosphate dehydrogenase
h	hours
Hb	Hemoglobin
HO	Heme Oxygenase
Hp	Haptoglobin
IL-10	Interleukin 10
IL-12	Interleukin 12
MBP	Mannose Binding Protein
µl	Microlitre
min	Minutes
ml	millilitre

mm	millimeter
mM	miliMole
M	Mole
MOMS	Mother Offspring Malaria Study
nm	nanomoles
ng	nanogram
NOS2	Nitric Oxide Synthase 2
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
rpm	Revolutions per minute
Th1	T helper 1 cells
TNFa	Tumor Necrotic Factor alpha
WHO	World Health Organisation

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Malaria is a vector-borne infectious disease caused by a protozoan parasite of the genus Plasmodium. Four species of the plasmodium parasite can infect humans; the most serious forms of the disease are caused by *Plasmodium falciparum* and *Plasmodium vivax*, but other related species (*Plasmodium ovale*, *Plasmodium malariae*) can also infect humans, and a fifth species *P. knowlesi* has recently been observed to naturally infect humans as a zoonosis in Southeast Asia. Each year, there are approximately 515 million cases of malaria, killing between one and three million people, the majority of whom are young children in Sub-Saharan Africa (Snow *et al.*, 2005). More than 90% of the 40 million Tanzanians are at risk of the disease which contributes to more than one third of the children deaths. Prevention is an important component of malaria control in endemic countries. It is achieved through vector control, personal protection measures like insecticide-treated bed nets and preventive treatment with anti-malarial drugs to vulnerable groups such as pregnant women who receive intermittent preventive treatment. Tanzania launched a national campaign in 2010 to sensitize the use of treated bed nets so as to combat the spread of the disease. The campaign is accomplished by distributing the nets at a subsidized price of Tsh 500/= for each child under five years and pregnant women. The aim is to eradicate the disease in the country by the year 2015. In terms of vaccination, the need is great but the biology is complex and the economics are disadvantageous although substantial efforts are in place to advance this effort with promising results.

The control of malaria is complicated by the emergence of drug resistance and the lack of an effective vaccine. The use of the treated bed nets and control of the mosquito breeding

sites is hindered not only by economic disabilities, but also by some social and educational matters. Children and pregnant women have been shown to be the most susceptible groups to the disease. It is known that some human genetic factors play a key role in disease susceptibility, progression and outcome. These include red blood cell polymorphisms like ABO blood group, sickle-cell trait (Hill *et al.*, 1991), G6PD deficiency as well as point mutations in the mannose binding protein (MBP) and in the promoter regions of both the TNFa and NOS2 genes. Other genetic factors shown to influence susceptibility to malaria are Haptoglobin and Heme Oxygenase-1 polymorphisms. The two proteins (Haptoglobin and Heme Oxygenase-1) have a key role in malaria pathogenesis being involved in free hemoglobin and heme breakdown, respectively. Haptoglobin has been shown to stimulate Heme Oxygenase-1 expression during malaria pathogenesis cascade.

Haptoglobin (Hp) is an acute phase protein that binds haemoglobin released during the intravascular lysis of erythrocytes. Cell free plasma haemoglobin is a potent pro-oxidant and haptoglobin is thought to be important in removing it from circulation and recycling the iron component for erythropoiesis via the reticulo-endothelial system and binding of cell surface receptor CD163 on circulating monocytes and macrophages and in the plasma. Under normal conditions up to 10% of haem iron may be recycled in this way, whilst the rest is via erythrophagocytosis of senescent red cells. Under conditions of haemolysis this proportion may be significantly increased (Andrews 2000). In humans, Hp is polymorphic with two co-dominant alleles, Hp^{1} and Hp^{2} encoded by a single gene on chromosome 16, resulting in three phenotypes Hp^{1-1}, Hp^{1-2} and Hp^{2-2}. Hp polymorphisms have been associated with a variety of different functional capacities and outcomes (Langlois & Delanghe, 2006) including haemoglobin binding affinity (Okazaki & Nagai, 1997; Okazaki *et al*., 1997), markers of oxidant stress (Langlois & Delanghe,

2006; Langlois *et al*., 1997; Asleh *et al.,* 2005), iron delocalisation within monocytes (Langlois *et al*., 2000) and immune regulation (Arredouani *et al*., 2003; Philippidis *et al*., 2004; Guetta *et al*., 2006). Moreover, Hp polymorphisms have been associated with a range of disease outcomes, including contradictory evidence for a protective effect of the Hp^2 allele against clinical malaria (Singh *et al*., 1986; Elagib *et al*., 1998; Quaye *et al*., 2000; Aucan *et al*., 2006; Quaye, 2003; Minang *et al*., 2004; Bienzle *et al*., 2005; Atkinson *et al*., 2007; Cox *et al*., 2007).

The action of Haptoglobin starts when red blood cells are broken down to release free hemoglobin (Hb). Haptoglobin binds the free Hb to form Hp:Hb complex. The complex binds to the CD163 receptor on the circulating macrophages and monocytes and this facilitates endocytosis of the complex. After endocytosis of Hb:Hp, the heme subunit of Hb is degraded by the rate-limiting heme oxygenase (HO) enzymes. Two main isoforms of HO have been characterized, with HO-2 being constitutively present under physiological conditions and HO-1 being inducible. The breakdown of heme yields biliverdin, free iron, and the carbon monoxide (CO) molecule, which has anti-inflammatory and cytoprotective effects. Haptoglobin in the Hp:Hb complex stimulates the HO-1 release. Different polymorphic forms of Haptoglobin are thought to have different stimulatory capacities for Heme Oxygenase-1. The level of Heme Oxygenase-1 expressed during malaria infection, which is also determined by Heme Oxygenase-1 gene promoter genotype affects the development of the disease. The hypothesis of the current study was that; Haptoglobin genotype influence susceptibility to Malaria in children and the influence of malaria to the Haptoglobin level is affected by Haptoglobin genotype.

1.2 Objectives

1.2.1 Main objective

To study relationship between human genetic factor; Haptoglobin and Malaria infection and pathogenesis.

1.2.2 Specific objectives

1.2.2.1. To study the influence of Haptoglobin Genotype on frequency of Childhood Parasitemia and severity of disease.

1.2.2.2. To study relationships of Malaria Parasitemia and Disease to plasma Haptoglobin level in relation to Haptoglobin genotype in children under five years

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Genetic basis to Malaria susceptibility

For some years it has been clear that the genetic determination of malaria susceptibility in humans is highly polygenic, as it is for many infectious diseases (Hill, 1986). Genetic polymorphisms of the innate immune system and of the erythrocyte have namely been proposed as factors protecting against severe malaria. Better knowledge of the polymorphic host genes associated with resistance to clinical malaria and/or with high parasite densities might provide new insights into disease mechanisms, and suggest new approaches for prophylactic or therapeutic interventions. In humans, malaria provides a clear example of host genetic factors influencing the onset, progression, type of disease developed, and ultimate outcome of infection (Hill, 1986).

2.2 Biology and function of Haptoglobin

Haptoglobin is an acute phase protein that is normally present in plasma at a concentration of 0.3–2 mg ml^{-1}, although levels may increase to 8 mg/ml during the acute phase response (Dobryszycka, 2007). It occurs in three phenotypes, the globular Hp^{1-1}, the linear polymer Hp^{1-2} and the circular polymer Hp^{2-2}, only one form being found in an individual. Haptoglobin binds specifically and irreversibly to free haemoglobin and prevents haemoglobin-initiated oxidative damage in renal tissue (Lim *et al.,* 2000), Hp^{2-2} and Hp^{1-2} binding less haemoglobin than Hp^{1-1} (Delanghe *et al.,* 2000). In recent years, haptoglobin has been found to have various other functions, e.g. inhibition of prostaglandin synthesis (Saeed *et al.,* 2005) promotion of angiogenesis (Cid *et al.,* 1993), antibody-like agglutination of *Streptococcus pyogenes* strains carrying the membrane

antigen, T4 (Ouyang *et al.*, 2007), inhibition of lectin-induced lymphocyte transformation, mediated by binding to CD22 (Baseler *et al.*, 2006), promoting Th1-dominant T cell responses, possibly mediated via CD11b (Arredouani *et al.*, 2003) and inhibition of the respiratory burst by phagocytes, mediated via CD11b (Langlois *et al.*, 2006).

Cell free plasma haemoglobin is a potent pro-oxidant (Langlois& Delanghe, 2006) and Haptoglobin is thought to be important in removing it from circulation and recycling the iron component for erythropoiesis via the reticulo-endothelial system and binding of cell surface receptor CD163 on circulating monocytes and macrophages (Kristiansen *et al.*, 2001). Humans differ from other mammalians by having different phenotypes of Hp, as based on two variant Hp alleles encoding Hp^1 and Hp^2 respectively (Langlois& Delanghe, 1996). Persons homozygous for Hp^1 form dimers (Hp^{1-1}), whereas persons homozygous for Hp^2 form oligomers (trimers and higher). The Hp^2 gene has been formed by a partial duplication of the region harbouring the cysteine involved in homodimerization. This extra cysteine residue can form disulfide bridges to other Hp^2 molecules, leading to a wide range of oligomers. Persons heterozygous for the two genotypes display the 2-1 phenotype, which is a mixture of dimers and oligomers (Langlois& Delanghe, 1996).

2.3 Haptoglobin and Malaria

Haptoglobin genotype and phenotype influence susceptibility to malaria and malaria parasitemia (Jacob *et al*., 2004; Quaye *et al*., 2000; Elagib *et al*, 2000; Atkinson *et al.*, 2006; Atkinson *et al.*, 2007). Hp polymorphisms have been associated with a range of disease outcomes, (reviewed by McDermid & Prentice, 2006), including contradictory

evidence for a protective effect of the Hp^{2} allele against clinical malaria (Singh *et al.*, 1986; Elagib *et al.*, 2000; Quaye *et al.*, 2000; Aucan *et al.*, 2006; Quaye, 2003; Mining *et al.*, 2004; Bienzle *et al.*, 2005; Atkinson *et al.*, 2006; Atkinson *et al.*, 2007; Cox *et al.*, 2007). In a study conducted in Ghana, the Hp^{1-1} phenotype was associated with susceptibility to *P. falciparum* malaria in general, and to the development of severe disease in particular (Quaye *et al.*, 2000) while in Kenya it was found that Hp^{2-2} genotype is associated with a reduced incidence of *P. falciparum* Malaria (Atkinson *et al.*, 2007). In an earlier study in Gambia, Hp^{2-2} genotype was a risk factor for anaemia in children in a malaria-endemic area, speculatively due to the reduced ability of the Hp^{2-2} polymer to scavenge free haemoglobin-iron following Malaria-induced haemolysis (Atkinson *et al.*, 2006). Functional differences appear between the Hp genotypes in terms of how well the Hp molecule prevents Hb induced oxidation, with the Hp^{1-1} protein being a superior antioxidant to the Hp^{2-2} protein (Frank *et al.*, 2001; Asleh *et al.*, 2003; Asleh *et al.*, 2005). Amplifying the magnitude of the disparity in antioxidant protection provided by Hp^{1-1} and Hp^{2-2}, the Hp^{1-1}/Hb complex is scavenged much more rapidly than the Hp^{2-2}/Hb complex by the CD163 pathway (Asleh *et al.*, 2003). The different and sometimes contradictory relationships between Haptoglobin genotype and malaria outcomes seen in earlier studies might be due to interactions with other genetic variants.

CHAPTER THREE

3.0 PATIENTS AND METHODS

3.1 Study site

The study was conducted in Morogoro, an eastern region of Tanzania. Samples were collected from two sites; Morogoro Regional hospital, where patients from urban and peri-urban areas were involved and Melela,a rural health centre located 50 km away from Morogoro Regional hospital. Tanzanian Entomological Inoculation Rate (EIR) has been estimated to be amongst the highest recorded (mean 367 ib/person/year, range 94–667), from studies conducted in rural areas (Hay *et al*., 2001), whereby lower EIRs (7.1-45.8 ib/person/year) have been shown for urban and peri-urban areas (Roberts *et al*., 2003). Morogoro town is located at 6°83'S 37°65'E and it is about 526m above see level. It experiences a tropical climate characterized by one main rain season (March-May) and a minor one between November and December with an annual rainfall range of 1200mm to 1800mm. Temperature ranges between 18°C to 34°C with the upper readings occurring during rain seasons between December and April.

3.2 Sampling

The study was conducted on a cohort design and was carried under an ongoing Mother Offspring Malaria Studies (MOMS) project studying malaria in pregnant mothers and children up to 5 years of age. Mothers who consent to participate, after adequate sensitisation on the project objectives and possible benefits, are enrolled to the study for themselves (if pregnant) or for their babies. The mothers are scheduled to attend (with babies) after every four weeks for clinical check up and sampling at the project clinics. However, in case a baby shows any sign of being sick, the mother is advised to take

him/her to the clinic even before the scheduled day. During clinic visits any child diagnosed for any clinical condition is treated accordingly. Routinely, blood collection is done in vacutainer tubes with EDTA by venipucture. The blood is taken for laboratory analysis while a portion of it is stored for future use. All the samples are stored at the MOMS project laboratory at the Morogoro regional hospital. For the purpose of this study, a cohort of 344 children was purposively selected (Table 1). Selection criteria aimed at meeting the objectives of this study. For that case, children with not more than five years of age, with at least 6 months of follow up, and at least one positive malaria parasitemia record were selected. Sample size was worked using a computer software G*Power 3.0 considering the findings of previous studies relating Haptoglobin and Malaria (Atkinson *et al*., 2007, Atif *et al*., 1998).

Table 1: Characteristics and number of the Children that were involved in the study

Number of patients	344
Female count	147
Male count	197
Mean Age during recruitment (years)	1.05
Study years	542.8
Mean study duration per patient (months)	18

3.3 Sample analysis and data collection

3.3.1 Parasite detection

Thick blood smears were made and stained with Giemsa for parasitological examination by microscopy. The parasites detected were scored against 200 white blood cells and the parasite densities calculated.

3.3.2 Haptoglobin genotyping

Genomic DNA was extracted from peripheral blood (Dry Blood Spots) using the QIAamp DNA Blood Mini Kit as suggested by the supplier (Qiagen, USA). The resulting DNA which was suspended in 150µl of dionized water was concentrated by drying in a speedvac, and then re-eluted in 10 µl. Genotyping was done by amplifying the Haptoglobin gene by Polymerase Chain Reaction (PCR) then separating the PCR products in 0.7% agarose gels. The PCR protocol was adopted from a study by Koch *et al*. (2003). Oligonucleotide primers A 5'-GAGGGGAGCTTGCCTTTCCATTG-3' as a sense primer and B 5'- GAGATTTTTGAGCCCTGGCTGGT-3' as anti-sense primer were used for amplification of a 1757-bp Hp^1 allele-specific sequence and a 3481-bp Hp^2 allele-specific sequence. Primers C 5'-CCTGCCTCGTATTAACTGCACCAT-3' (sense) and D 5'-CCGAGTGCTCCACATAGCCATGT-3' (anti-sense) were used to amplify a 349-bp Hp^2 allele-specific sequence. In Hp^1 and Hp^2, the annealing sites for primer A are located immediately upstream of the 1711-bp unit and the 1724-bp unit, respectively. The nucleotide at the 5' end of primer A corresponds to position 188639 in AC004682 (Hp^1) and position 2781 in M69197 (Hp^2). Primer B has binding sites just downstream of the 1711-bp elements of Hp^1 and Hp^2. The nucleotide at the 5' end of primer B corresponds to position 186883 in AC004682 (Hp1) and position 6261 in M69197 (Hp^2). Depending on the genotype represented by the template DNA, an Hp1-specific product of 1757 bp and/or an Hp^2-specific product of 3481 bp are generated in PCRs with primers A and B. Primers C and D have one binding site in allele Hp1 and two binding sites in allele Hp^2. In reactions with primers C and D, a PCR product, 349 bp in length, is generated only in the presence of the Hp^2 template, whereas no product is formed in the presence of the Hp^1 template. This kind of allelic specificity is attributable to the relative positions of the binding sites and the 5'→3' orientation of primers C and D. The template

for the 349 bp Hp^2-specific amplification product, including the annealing sites for primers C and D, extends from nucleotide position 4352 to 4700 in M69197. There is also one binding site each for primers C and D at the corresponding positions in the Haptoglobin-related gene, although the sequences of primers and annealing sites are not 100% complementary. Importantly, with the Haptoglobin-related gene as a template, amplification reactions with primers C and D proceed in opposite directions, which do not allow a PCR product to be generated

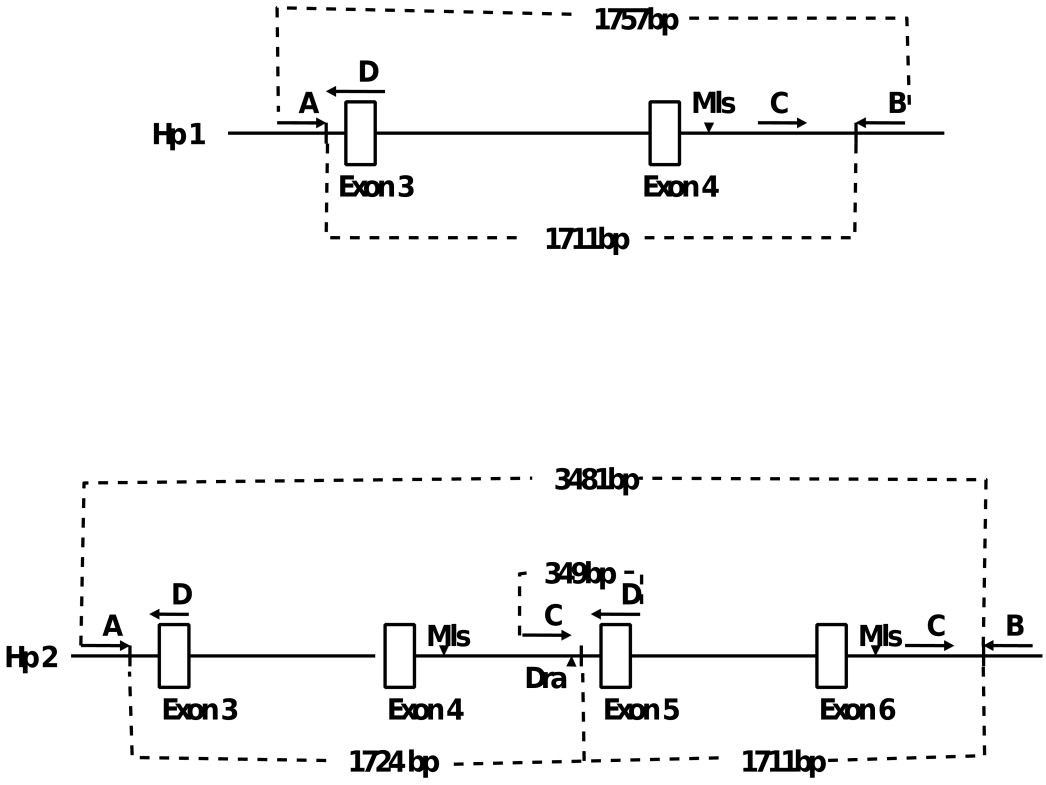

Figure 1: Partial structure of Haptoglobin alleles Hp^1 and Hp^2 (Koch *et al*., 2003)

In the above figure Hp^1 is represented by subtype Hp 1S, as indicated by the presence of the 1711 bp element. Hp^2 is represented by subtype Hp 2FS, as shown by the presence of the 1724 bp element followed by the 1711 bp element. The *arrows*, representing oligonucleotide primers A, B, C, and D, are located at positions next to the binding sites

of the primers within the DNA sequence. The directions of the arrows indicate the 5'→3' orientation of the primers relative to the template DNA. To allow for adequate illustration, the *arrows* shown are considerably longer in relation to the sizes of the primers they represent. Also shown are the sizes of the PCR products obtained with primer pairs A/B (1757 and 3481 bp) and C/D (349 bp). *Mls*, site for restriction enzyme *Mls*I; *Dra*, site for restriction enzyme *Dra*I located in the part of the Hp^{2} allele that is amplified with primers C and D.

The 30µL reactions contained 2.5 U of *Taq* polymerase (Qiagen), 50–100 ng of DNA, and 600 µM each of dATP, dCTP, dGTP, and dTTP (Roche); PCR buffer was used as recommended by the supplier (Applied Biosystems) with no supplements added. After initial denaturation at 95°C for 2 min, the two-step thermocycling procedure consisted of denaturation at 95°C for 1 min and annealing and extension at 69 °C for 2 min (in the presence of primers A and B) or 1 min (in the presence of primers C and D), repeated for 35 cycles, and followed by a final extension at 72 °C for 7 min. Two reactions per sample were carried using the two sets of the primers used separately. For this case, an individual was recorded to have Hp^{1-1} genotype if the 1711 bp band only is seen in PCR product of primer set A&B and no band seen with primer sets C&D. Similarly, a PCR product of primer set C&D was recorded as genotype Hp^{2-2} if the 349 bp band only is seen and no band seen with primer set A&B. However, if a sample yields both 1711 bp band and 349 bp band in the two reactions using primer sets A&B and C&D respectively, it was recorded as Hp^{1-2} genotype.

Plate 1: Thermocycler machines used for PCR

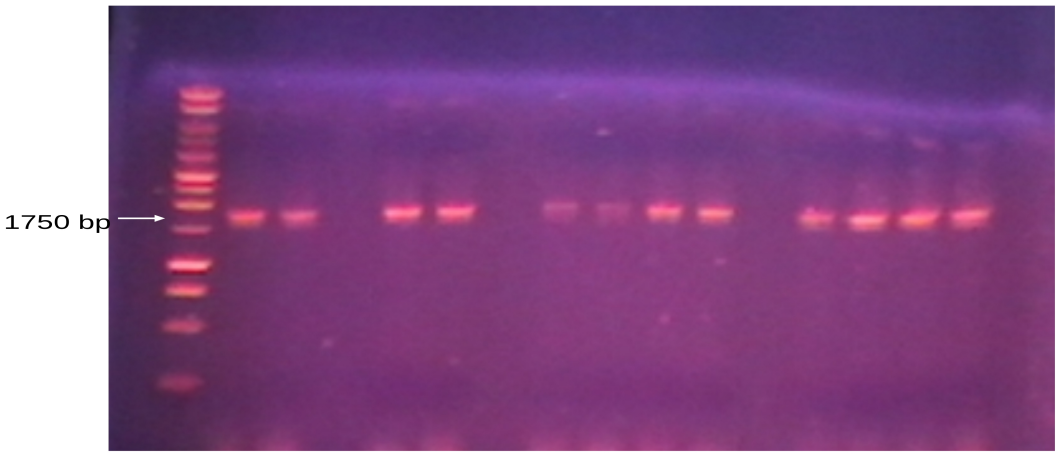

Plate 2: Photographic presentation of an Agarose gel showin the 1750 bp fragment of the Hp^{1} gene.

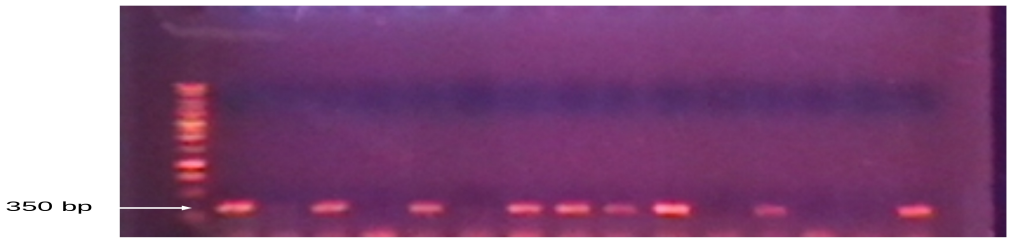

Plate 3: Photographic presentation of an Agarose gel with PCR product displayed over a UV light box. The 350 bp band is for the Hp^2 gene.

3.3.5 Quantification of Plasma Haptoglobin

Plasma Hp levels were determined by an enzyme-linked immunosorbent assay (ELISA) using 96-well plates coated with chicken anti-human Hp (GenWay) as capture antibody and monoclonal anti-human Hp (GenWay) as detection antibody. Immunoplates (Immulon, USA) were coated overnight at 4°C with the capture antibody diluted in coating buffer (0.05 M Carbonate-Bicarbonate, pH 9.6) to a final concentration of 5 µg/mL (100 µL/well). Wells were washed three times with 200 µL/well of wash buffer (0.05% Tween in PBS pH 7.4). Non fat dairy milk (1%) in PBS was used to block the wells (200 µL/well for 1 hour at room temperature), followed by three washes as described above. Samples were diluted 1:1,000 in sample diluents (50 mM Tris, 0.14 M Nacl, 1% BSA, 0.05% Tween 20) and Hp standards (500 ng/mL, 250 ng/mL, 125 ng/mL, 62.5 ng/mL, 31.2 ng/mL, 15.6 ng/mL and 7.8 ng/mL) prepared using pooled Hp (GenWay) in sample diluents (50 mM Tris, 0.14 M Nacl, 1% BSA, 0.05% Tween 20). Aliquots of 100 µL were added to wells in duplicate, incubated for 1 hour at room temperature, and washed five times in washing buffer. Detection was done by adding 100 µL of detection antibody in each well for 1 hour diluted in conjugate diluents (50 mM Tris, 0.14 M Nacl, 1% BSA, 0.05% Tween 20) to a concentration of 0.025 µg/mL. This

was followed by addition of substrate and incubation for 20 minutes. Optical densities were read at 405 nm using SOFTmax PRO spectrophotometer. A standard curve was plotted from which sample levels were extrapolated and multiplied by dilution factor, giving sample Hp concentrations.

CHAPTER FOUR

4.0 RESULTS

4.1 Haptoglobin Genotypes

4.1.1 General characteristics

A total of 345 patients were successfully genotyped for Haptoglobin. The general characteristics of the patients for the three Haptoglobin genotypes are shown in Table 2 below. As can be seen 85 (24.5%) patients were found to carry Hp^{1-1} genotype, whereas 88 patients (25.5%) carried Hp^{2-2} genotype and 171 patients (50%) had the Hp^{1-2} genotype. This distribution obeys the Hardy-Weinberg equilibrium. The mean ages of the patients during recruitment were 1.03 years, 1.07 years and 1.05 years for the Hp^{1-1}, Hp^{1-2} and Hp^{2-2} genotypes respectively and statistically these ages were not significantly different. Similarly the three genotype groups had non-significantly different mean study duration of 18, 19, and 18 months; equivalent to 127. 8, 276.1 and 138.9 study years for Hp^{1-1}, Hp^{1-2} and Hp^{2-2} respectively. The ratio of female: male was 0.77, 0.71 and 0.80 for the respective Hp^{1-1}, Hp^{1-2} and Hp^{2-2} genotype groups.

Table 2: General Characteristics of the Patients used in the study. Comparison of the average age in the three Haptoglobin genotype groups is done by Kruskal-Wallis test (*p=0.94*)

Parameter	Genotype			*p*- value
	Hp^{1-1}	Hp^{1-2}	Hp^{2-2}	
Number of patients	85	171	88	
Female count	37	71	39	
Male count	48	100	49	
Mean Age during recruitment (years)	1.03	1.07	1.05	*0.94*
Study years	127.8	276.1	138.9	
Mean study duration per patient (months)	18	19	18	

4.1.2 Malaria status in relation to Haptoglobin Genotype

The patients' Haptoglobin genotypes were related to the malaria status of the patients for the purpose of studying if genotype can influence the malaria situation of an individual. Malaria status was considered in terms of parasitemia and clinical characteristic of a given patient. For the case of parasitemia a sample was characterized either as positive or negative based on microscopic examination of blood smears. Positive samples were then characterized either as positive symptomatic or asymptomatic depending on the clinical examination of a particular patient during sample collection. Positive symptomatic samples were further grouped depending on the specific symptoms of the patient from which the type of malaria was referred. Therefore a symptomatic malaria case was referred as mild, complicated, severe or cerebral. As explained in methodology section, severe cases were either clinically severe or WHO severe depending on the criteria outlined. Comparison was made between the three Haptoglobin genotypes malaria status and the results are summarized in Table 3.

Table 3: Comparison of Malaria status and Haptoglobin genotypes

	Genotype			Unpaired t-test *p*-value			Kruska-Wallis
	Hp^{1-1}	Hp^{1-2}	Hp^{2-2}	Hp^{1-1}, Hp^{1-2}	Hp^{1-1}, Hp^{2-2}	Hp^{1-2}, Hp^{2-2}	test *p*- value
Total Slides	1 949	4 344	2 284				
Negative Slides	1 721	3 839	2 000				
Positive slides	228	505	284	*0.29*	*0.11*	*0.36*	*0.52*
Positive Asymptomatic	187	409	242	*0.38*	*0.07*	*0.16*	*0.43*
Positive Symptomatic	41	96	42	*0.47*	*0.96*	*0.43*	*0.74*
Mild	17	31	11	*0.76*	*0.22*	*0.34*	*0.71*
Complicated	12	29	13	*0.59*	*0.91*	*0.68*	*0.92*
WHO Severe	5	15	5	*0.51*	*0.96*	*0.46*	*0.95*
Clinically Severe	7	21	11	*0.33*	*0.36*	*0.96*	*0.85*
Cerebral	0	0	2				*0.95*

4.1.3 Parasitological characteristics of the patients

The patients made a total of 8 577 visits to the clinics, resulting into the same number of the blood samples collected. From each sample a blood slide was made and this is equivalent to 29 slides from each baby. As can be seen in Table 3 above, 1 949 slides were collected from 85 children with Hp^{1-1} genotype, 4344 slides were collected from 171 children with Hp^{1-2} genotype while 2284 slides were from 88 candidates with Hp^{2-2} genotype. All the slides were examined microscopically for malaria parasite and it was found that 1017 (11.9%) were positive giving an estimated average of 1 positive malaria parasitemia in every eight clinic visits. Comparison of the three Haptoglobin genotypes shows no significant difference in the distribution of the positive slides (*p*- value = *0.52*). It is seen in Table 4 below that 228 (11.7%) slides from Hp^{1-1} children were positive for malaria parasitemia while 505 (11.6%) and 284 (12.4%) were positive from Hp^{1-2} and Hp^{2-2} children respectively. Comparison of the positive and negative blood slides for the three genotypes is further illustrated in Fig. 2.

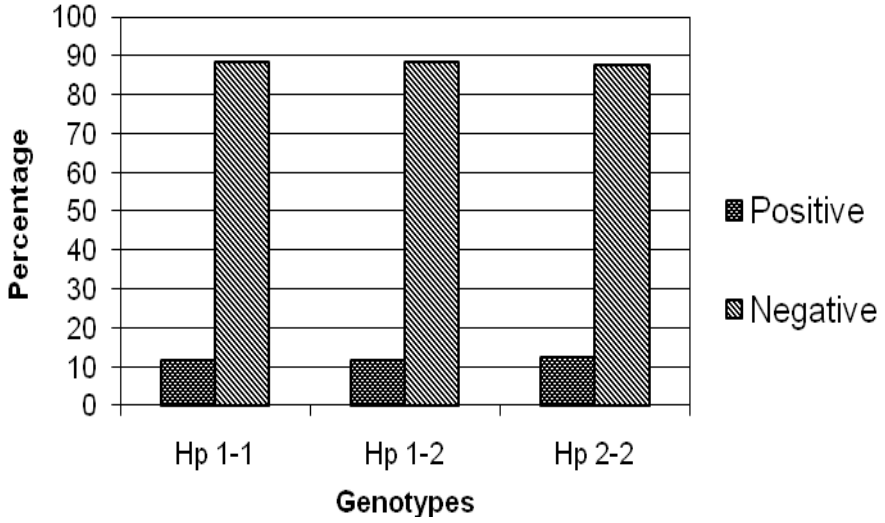

Figure 2: Percentage distribution of negative and positive blood slides in the three genotype groups

Table 4: Parasitological characteristics of the patients

	Genotype		
	Hp^{1-1}	Hp^{1-2}	Hp^{2-2}
	n= 85	n= 171	n= 88
Total slides	1 949	4 344	2 284
Positive slides (%*)	228 (11.7%)	505 (11.6%)	284 (12.4%)
Negative slides (%**)	1 721 (88.3%)	3 839 (88.4%)	2 000 (87.6%)

* Percentage of the positive slides per the total slides in the given genotype group

** Percentage of the negative slide per the total slides in the given genotype group

4.1.4 Frequency of Malaria positive cases

The total number of positive malaria parasitemia cases in each child ranged between 0-14. It was observed that there were only three children who didn't record any case of positive parasitemia for the whole study period. These children; two of them males and one female, were followed for 6, 16 and 17 months, respectively. As can be seen in Table 5 and illustrated in Fig. 2 below, majority of the children had 1-2 cases of positive malaria and their distribution by Hp genotype was 54.1%, 52.6% and 47.7% for Hp^{1-1}, Hp^{1-2} and Hp^{2-2} respectively. The frequency of the positive parasitemia cases was not significantly different when compared between the three Haptoglobin genotypes (Table 5).

Table 5: Frequency of Malaria positive cases of the patients in different Hp genotypes

The table indicates the number of children in the given group of frequency of occurrence and their percentage per total number of children in the given genotype group.

Frequency	Hp^{1-1}	Hp^{1-2}	Hp^{2-2}
None (%)	0 (0)	2(1.2)	1(1.1)
1 to 2(%)	46(54.1)	90(52.6)	42(47.7)
3 to 4(%)	26(30.6)	44(25.8)	31(35.2)
>4(%)	13(15.3)	35(20.4)	14(16)

4.1.5 Clinical characteristics of the patients

The clinical characteristics of the study patients and their distribution among the three Haptoglobin genotypes are summarized in Table 6 below. It was observed that there were a total of 1017 positive parasitemia cases but only 179 (17.6%) of these were accompanied by malaria symptoms. As it is shown in Table 6, the symptomatic cases were unevenly distributed among the three genotypes with 41 cases in Hp^{1-1}, 96 cases in Hp^{1-2} and 42 cases in Hp^{2-2}. When these cases were expressed over the total number of positive cases within each genotype, it was found that symptomatic cases were 18%, 19%, and 14.8% of all positive cases for genotypes Hp^{1-1}, Hp^{1-2} and Hp^{2-2} respectively. Similarly the distribution of the frequency of different types of symptomatic malaria was studied among the Hp genotypes. It was revealed that mild cases were 41.5% of all symptomatic cases in Hp^{1-1}, 32.3% and 26.2% in Hp^{1-2} and Hp^{2-2} genotypes respectively. Complicated cases constituted 29.2%, 30.2% and 30.9% of symptomatic cases for the respective Hp^{1-1}, Hp^{1-2} and Hp^{2-2} genotypes. Severe cases according to the WHO criteria constituted 12.2% of symptomatic cases in Hp^{1-1} genotype, 15.6% in Hp^{1-2} and 11.9% in Hp^{2-2}. By using the clinic criteria on the other hand, severe cases comprised 17.1% of

symptomatic cases in Hp^{1-1}, 21.9% and 26.2% in genotypes Hp^{1-2} and Hp^{2-2}, respectively. There were only two cases of cerebral malaria, both recorded in children with Hp^{2-2} genotypes (Table 6). The distribution of the Malaria types in symptomatic cases for the three genotypes is shown in Table 6 and further illustrated in Fig. 3.

Table 6: Total positive parasitemia cases, positive symptomatic cases and distribution of different symptomatic malaria types.

	Genotype		
	Hp^{1-1}	Hp^{1-2}	Hp^{2-2}
Total Positive slides	228	505	284
Positive Symptomatic (%*)	41(18)	96(19)	42(14.8)
Mild (%**)	17(41.5)	31(32.3)	11(26.2)
Complicated (%**)	12(29.2)	29(30.2)	13(30.9)
WHO Severe (%**)	5(12.2)	15(15.6)	5(11.9)
Clinically Severe (%**)	7(17.1)	21(21.9)	11(26.2)
Cerebral (%**)	0	0	2(4.8)

* Percentage of symptomatic cases per the number of positive cases in the given genotype group

** Percentage of a given malaria type per the number of symptomatic cases in the given genotype group.

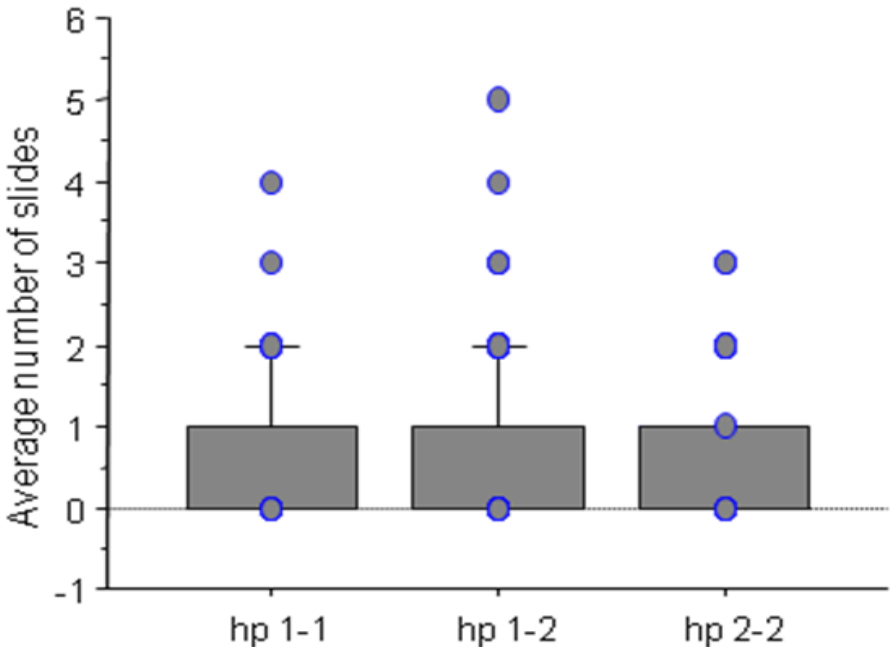

Figure 3: Frequency of different Malaria symptoms distributed by Hp genotype

4.1.6 Symptomatic and Asymptomatic Malaria cases

It was found that the total number and the average number of slides with symptomatic or asymptomatic cases didn't differ significantly between the three Haptoglobin genotypes. Fig. 4(a) and 4(b) show a comparison of number of slides with symptomatic and asymptomatic cases, respectively between the Haptoglobin genotypes Hp^{1-1}, Hp^{1-2} and Hp^{2-2}.

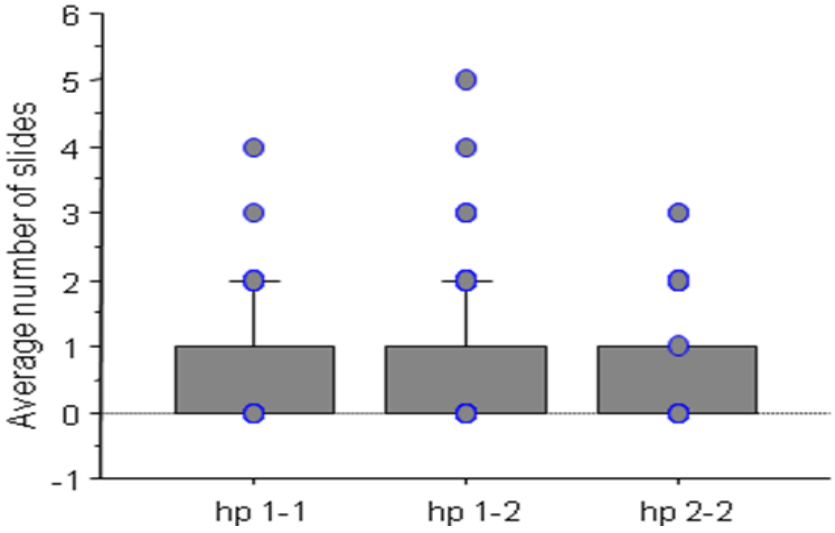

Figure 4a: Box plot presentation of the average number of slides from positive symptomatic cases per individual child for the three Haptoglobin genotypes.

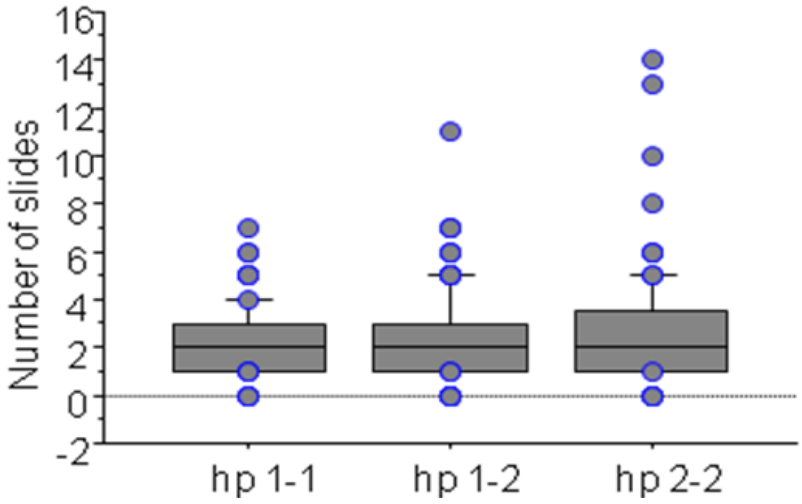

Figure 4b: Box plot presentation of the average number of slides from positive asymptomatic cases per individual child for the three Haptoglobin genotypes.

4.1.7 Mild Malaria cases

Mild Malaria contributed more of symptomatic cases in Hp^{1-1} (41.5%) and Hp^{1-2} (32.3%) genotype individuals. In Hp^{2-2} mild Malaria ranked second by accounting 30.2% of

symptomatic cases (Fig. 4). A total of 59 mild malaria cases were recorded in 52 children over the study duration. Out of these children, 47 suffered once, three suffered twice and two suffered thrice. Comparison of the 59 mild Malaria cases by Hp genotypes indicated that 17 were from Hp^{1-1} genotype, 31 from Hp^{1-2} and 11 from Hp^{2-2}. This is depicted in Fig. 5.

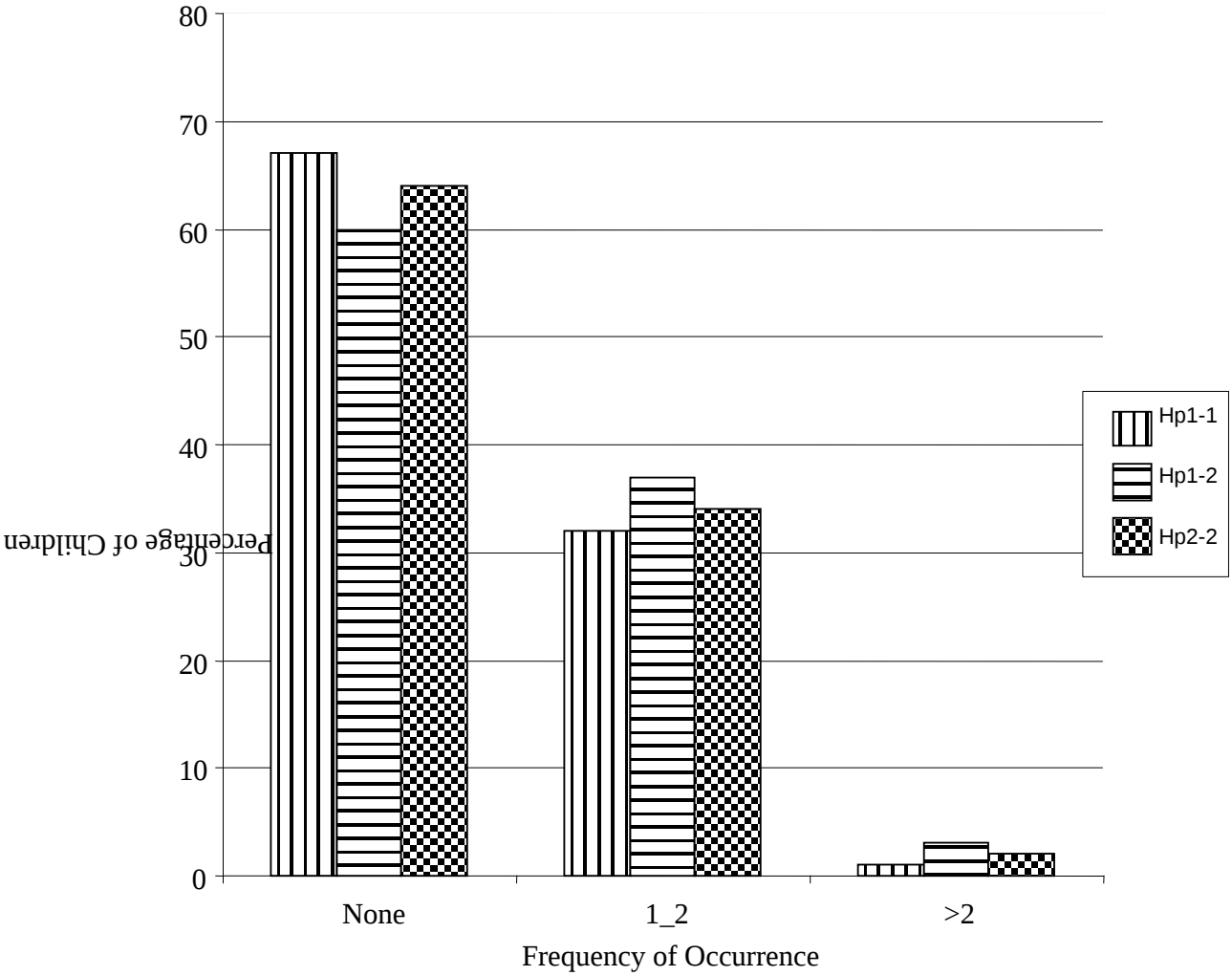

Figure 5: Histogram presentation of frequency of mild symptomatic cases for the three Haptoglobin genotypes. It was shown that children recorded 0-3 mild symptomatic cases over the study period.

4.1.8 Complicated Malaria

There were 54 cases of complicated malaria recorded over the study duration accounting for 23% of all symptomatic cases. These cases were recorded in 50 children, whereby 45 children experienced one complicated case, and 5 children had two cases. Complicated malaria ranked the second in frequency of occurrence in Hp^{1-1} and Hp^{1-2} individuals contributing 20.2% and 30.2% of all symptomatic cases, respectively. On the other hand, complicated malaria ranked first in occurrence contributing about 30.9 of all cases in Hp^{2-2} individuals. As can be seen in Fig. 6, majority of complicated cases occurred except in few children who had two complicated cases recorded.

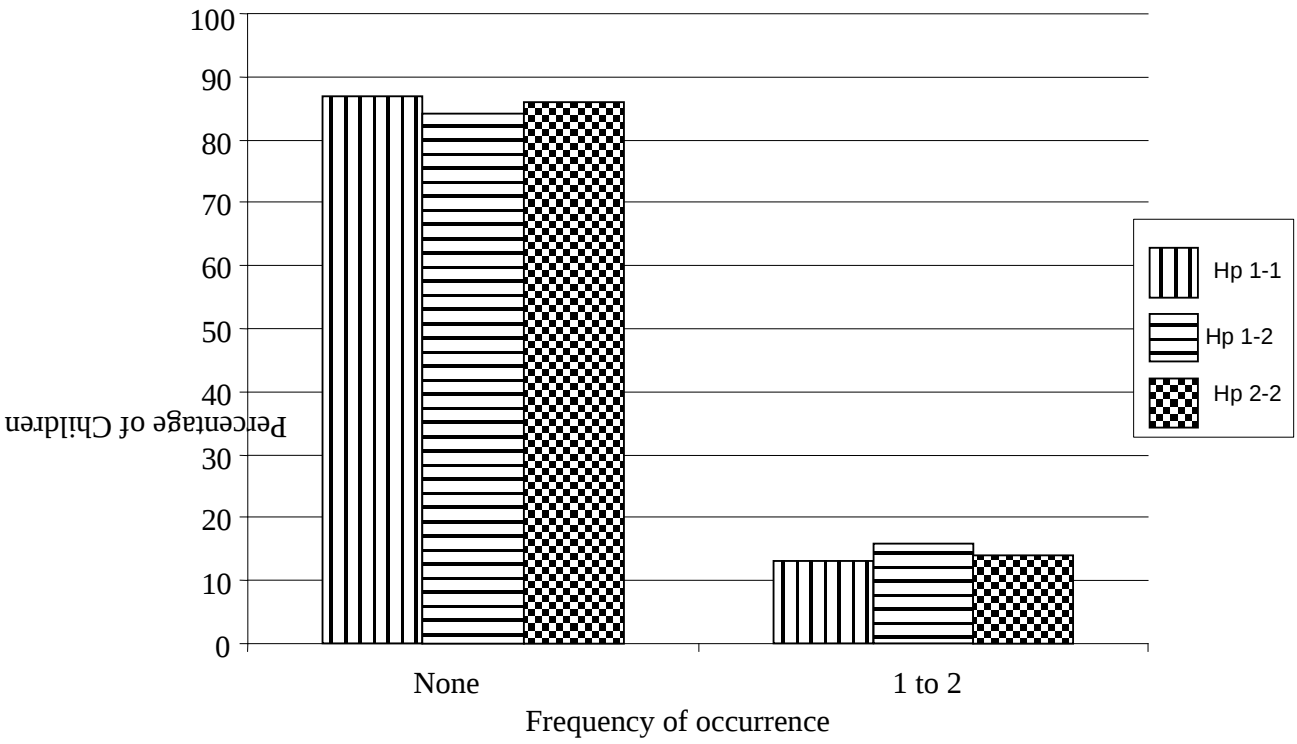

Figure 6: Histogram presentation of frequency of complicated cases for the three Haptoglobin genotypes. It was shown that children recorded 0-2 complicated Malaria cases over the study period.

4.1.9 Clinically Severe Malaria

A total of 39 cases of clinically severe malaria were recorded, which accounted for 22% of all symptomatic cases. These cases were recorded from 39 different children each with one case. Clinically severe cases were 17.1% of all symptomatic cases in Hp^{1-1} genotype children, 21.9% in Hp^{1-2} and 26.2% in Hp^{2-2}. Fig. 7 illustrates the number of clinically severe cases recorded in the three Haptoglobin genotypes.

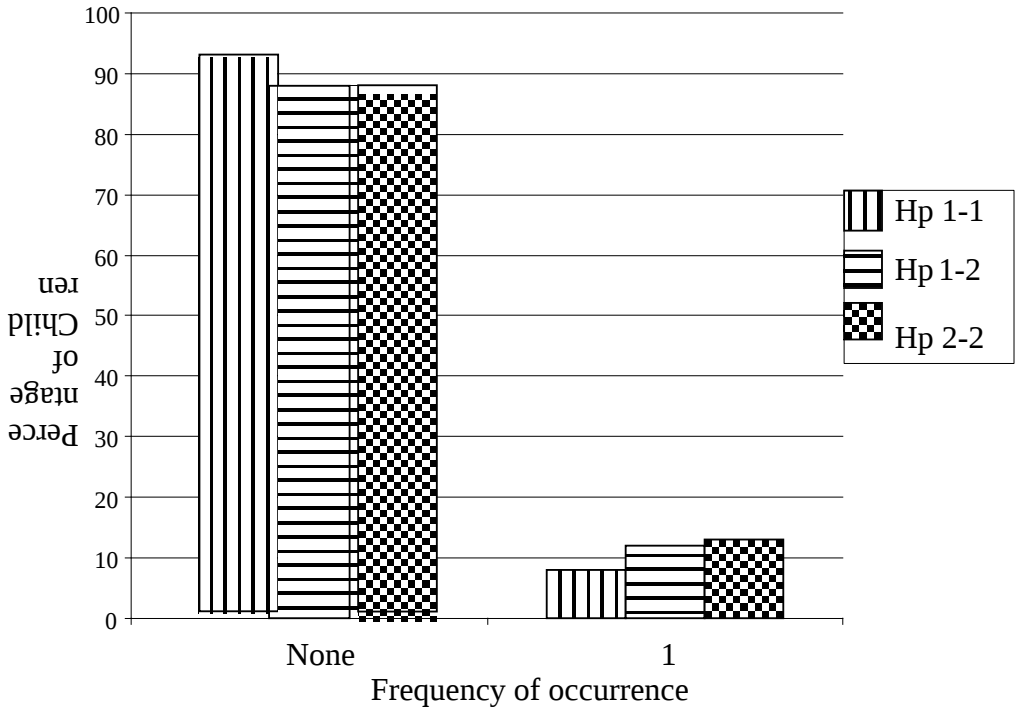

Figure 7: Histogram presentation of frequency of clinically severe malaria cases for the three Haptoglobin genotypes. It was shown that children recorded 0-1 clinically severe Malaria cases over the study period.

4.1.10 WHO severe malaria

A total of 25 cases recorded were classified as severe according to WHO criteria and these constituted 14% of all symptomatic cases. The cases were recorded in 21 children whereby 18 of them had one case each, two had two cases each and one had three cases

of severe malaria over the study duration. It was observed that this type of malaria contributed about 12.2% of all cases in Hp^{1-1} genotype, 15.6% in Hp^{1-2} and 11.9% in Hp^{2-2}. Fig. 8 below shows the distribution of the number of WHO cases in the three Haptoglobin genotypes.

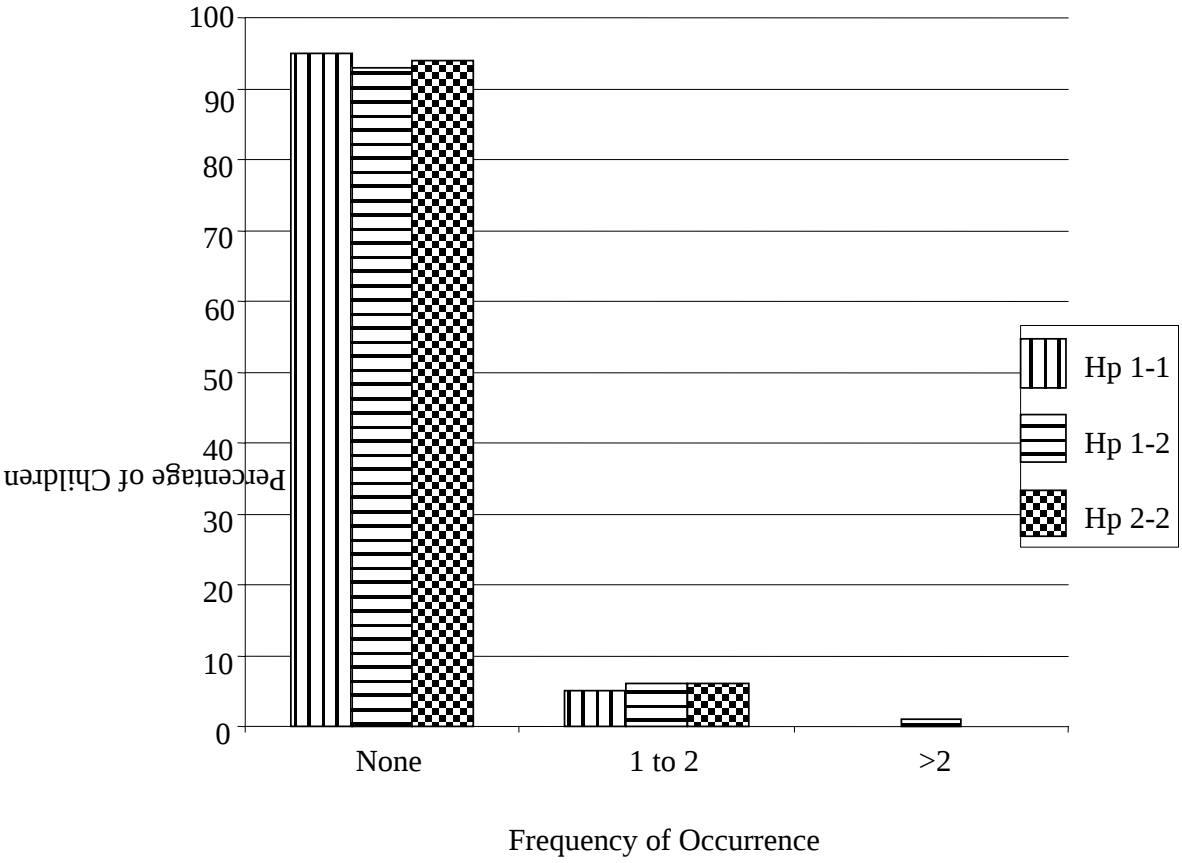

Figure 8: Box plot presentation of the average number of slides from WHO severe cases per individual child for the three Haptoglobin genotypes. The plot shows that children recorded 0-3 WHO severe cases over the study period.

4.1.11 Cerebral Malaria

There were only two cases of cerebral malaria during the study. These cases were recorded from two children of genotype Hp^{2-2} and they represented 4.8% of all symptomatic cases.

4.2 Plasma Haptoglobin level and Malaria

4.2.1 General characteristics of the patients

A total of 205 blood samples were measured for plasma Haptoglobin level. Out of these 101 were blood smear positive, whereas 104 were negative for Malaria parasites as determined by microscopy. There were 66 pairs of positive and negative samples each from one individual. All three Haptoglobin genotypes were involved, whereas 58 individuals were Hp^{1-1} genotype, 94 were Hp^{1-2} genotype and 53 samples were Hp^{2-2} genotype (Table 7). As shown in the table, there were 31, 43 and 27 positive samples representing the genotypes Hp^{1-1}, Hp^{1-2} and Hp^{2-2} respectively. Conversely there were 27, 51 and 26 samples of the Hp^{1-1}, Hp^{1-2} and Hp^{2-2} genotypes respectively.

Table 7: Number of the samples used for determining plasma Haptoglobin level

	Hp^{1-1}	Hp^{1-2}	Hp^{2-2}	Total
Positive parasitemia	31	43	27	101
Negative parasitemia	27	51	26	104
Total	58	94	53	205

4.2.2 Plasma Haptoglobin level in relation to Haptoglobin genotype and Malaria status

The plasma Haptoglobin levels for the three genotypes were found to differ both in Malaria positive and negative individuals. As can be seen in Table 8, parasite negative individuals had plasma Haptoglobin level ranging from 29.4 µg/ml to 496.9 µg/ml and a mean value of 180.7 µg/ml for Hp^{1-1} genotype individuals. Hp^{1-2} children had plasma Hp levels ranging from 9.5 µg/ml to 458.6 µg/ml with the mean value of 203.9 µg/ml. Children with genotype Hp^{2-2} had plasma Haptoglobin level ranging from 91.8 µg/ml to 500.0 µg/ml and a mean value of 245.7 µg/ml. With Kruskal-Wallis test, the plasma Haptoglobin levels in the negative samples significantly differed between the three

genotypes (*p*- value 0.03), being higher in Hp^{2-2} individuals. When compared by unpaired t-test, plasma Haptoglobin level for negative samples is neither significantly different between Hp^{1-1} and Hp^{1-2} (*p*- value 0.35) nor is it different between Hp^{1-2} and Hp^{2-2} (p-value 0.08). However, there is significant difference when plasma Haptoglobin levels of Hp^{1-1} and Hp^{2-2} are compared by the same test (*p*- value 0.01). On the other hand the mean (range) plasma Haptoglobin levels for Malaria positive individuals were 170.8 µg/ml (10.6 µg/ml - 474.9 µg/ml), 167.5 µg/ml (14.4µg/ml - 330.8 µg/ml) and 133 µg/ml (8.9 µg/ml - 325.3 µg/ml) for genotypes Hp^{1-1}, Hp^{1-2} and Hp^{2-2}, respectively (Fig. 9 a – c). The variation in these values for the three genotypes is not significantly different when compared together or when compared one by one with unpaired t-test (Table 8).

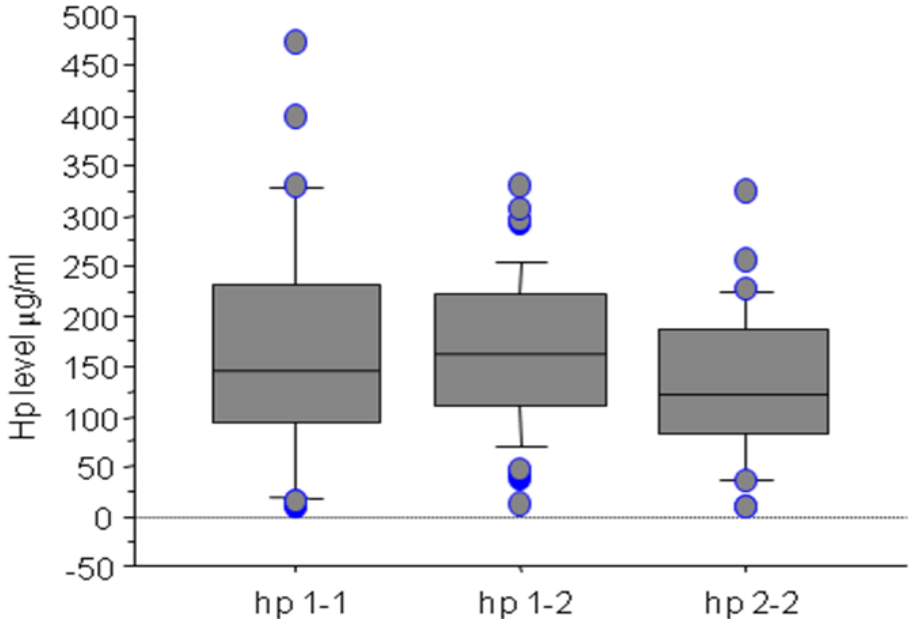

Figure 9a: Box plot for Plasma Haptoglobin levels in samples from children with positive parasitemia. In majority of the samples, the level ranged between 100 – 130 µg/ml.

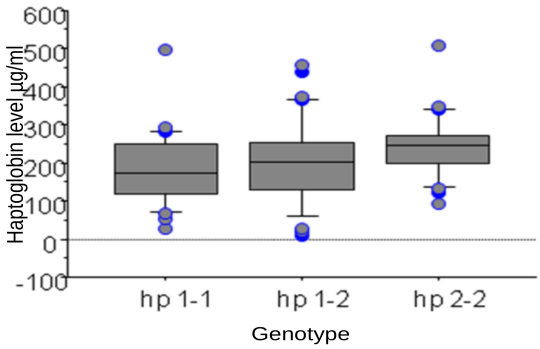

Figure 9b: Box plot for Plasma Haptoglobin levels in samples from children with negative parasitemia. In majority of the samples, the level ranged between 130 – 270 µg/ml.

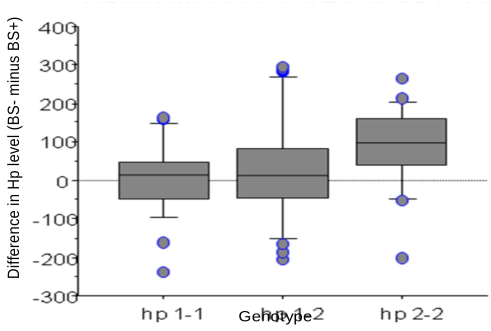

Figure 9c: Box plot for Difference in Plasma Haptoglobin levels between a pair of a negative and a positive sample from an individual child (level in BS- sample minus level in Bs+ sample). Comparison with unpaired t-test shows the difference to be significantly higher in Hp^{2-2} ($p=0.03$) when compared with Hp^{1-1}.

The mean Haptoglobin level for all negative samples was found to be higher (208.3 µg/ml) when compared to the positive samples (159.3 µg/ml) giving a difference of 49 µg/ml. As can be seen in Fig. 10, when the difference in Hp level is broken into particular genotypes, the difference is higher in Hp $^{2-2}$ (83.2 µg/ml) and lower in Hp1-1 (8.8 µg/ml), while in Hp $^{1-2}$ it remains intermediate (31.8 µg/ml). These differences when compared together by Kruskal-Wallis test show no statistically significant difference (*p*-value 0.07), but with unpaired t-test, the difference between Hp^{1-1} and Hp^{2-2} is significant (*p*-value 0.03). The plasma Haptoglobin levels in relation to Haptoglobin genotype are summarized in Table 8.

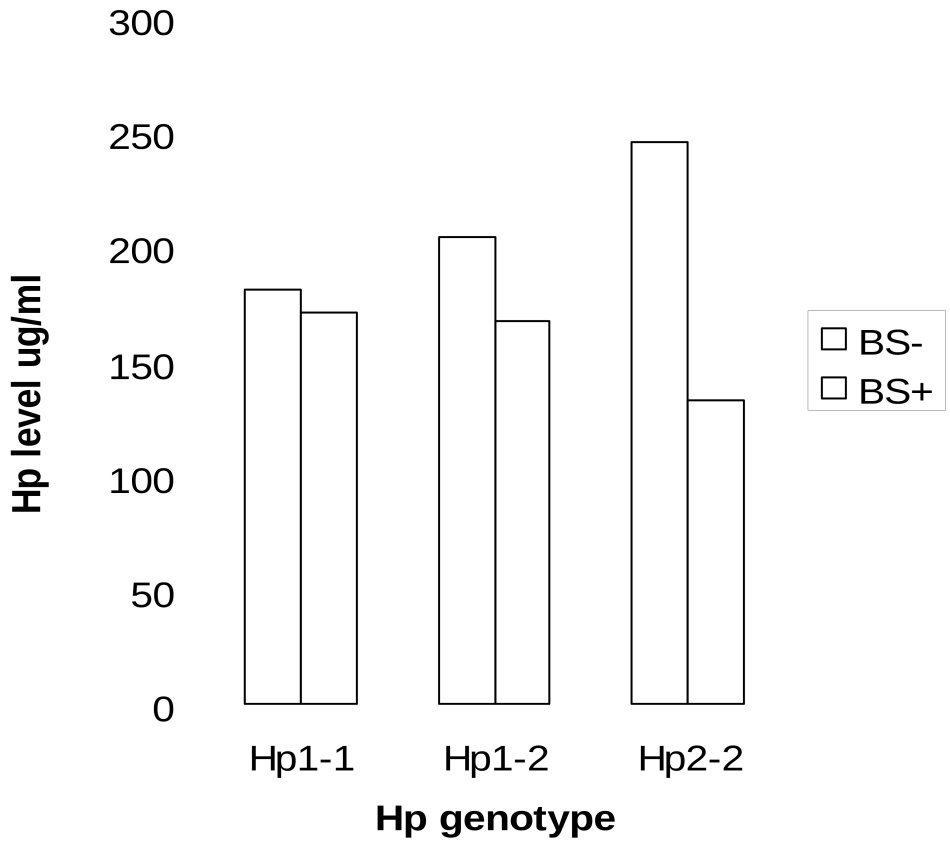

Figure 10: Relative difference in Plasma Hp level between Malaria negative and Malaria positive samples

Table 8: Plasma Hp levels and difference between positive and negative samples in the three Haptoglobin genotypes

	Hp^{1-1}	Hp^{1-2}	Hp^{2-2}	Unpaired t-test *p*-value			Kruskal-Wallis test p-value
				Hp^{1-1}, Hp^{1-2}	Hp^{1-1}, Hp^{2-2}	Hp^{1-2}, Hp^{2-2}	
Mean Hp level, BS+ (range)	170.8 µg/ml (10.6-474.9)	167.5 µg/ml (14.4-330.8)	133 µg/ml (8.9-325.3)	0.88	0.15	0.07	0.19
Mean HP level BS- (range)	180.7 µg/ml (29.4-496.9)	203.9 µg/ml (9.5-458.6)	245.7 µg/ml (91.8-509.1)	0.35	0.01	0.08	0.03
Mean Difference between Hp Level of BS- and BS+ samples	8.8 µg/ml	31.8 µg/ml	83.2 µg/ml	0.51	0.03	0.18	0.07

CHAPTER FIVE

5. DISCUSSION

5.1 Haptoglobin genotype and Malaria

The distribution of the three Haptoglobin genotypes occurs in the ratio 1:2:1 for Hp^{1-1}: Hp^{1-2}: Hp^{2-2}. This distribution is in Hardy-Weinberg equilibrium and it is consistent with another study done in Coastal Kenya (Atkinson *et al.*, 2007). Cumulative evidence indicates that the distribution of the different Hp phenotypes within a population is controlled by genetic and environmental factors (Smithes, 1955; Allison *et al*., 1958). Therefore data from the present study strongly support that genetic pool of a given population is greatly influenced by environmental selective factors.

It can be estimated from present study that parasitemia prevalence for the study population is 12%. The National Bureau of Statistics in Tanzania released a report in 2009 that estimated malaria prevalence in Morogoro to be 16%. The reason for this might have occurred by chance but also due to the difference in nature of the methodology used. The present study was conducted in children less than five years, an age group known to be more vulnerable to malaria infection. However, the study was designed to allow study patients to have regular malaria check-ups and appropriate treatment whenever necessary. This could have led to reduced parasite prevalence in the study group as compared to what can be happening in the community. Another reason may be due to the fact that the study group is relatively more naïve to malaria transmissions when compared to the whole community making them more prone to disease development, hence more likely to get treated upon infection.

Several previous studies have demonstrated that Hp^{2-2} genotype is associated with reduced susceptibility to malaria. In the study by Quaye *et al.* (2000) it was found that Hp^{2-2} phenotype was significantly less presented in malaria patients as well as in

complications of malaria disease. Previously, Elagib *et al.* (1998) conducted a similar study in Ghana with results suggesting that the Haptoglobin phenotype l-l is associated with susceptibility to falciparum malaria and the development of severe complications; alternatively, the other phenotypes may confer resistance. Atkinson *et al*. (2006) found in a study conducted in Kenya that Hp^{2-2} genotype was associated with reduced episodes of clinical malaria. One year before, another study by Bienzle *et al*. (2005) in northern Ghana found a limited influence of Haptoglobin genotype to malaria susceptibility. The findings in these studies although somehow contradicting, mostly show a relation between Hp^{2-2} and reduced risk of malaria infection and development of the disease. In the present study none of the Hp genotypes *per se* was found to increase the risk of malaria parasitemia or development of the disease. Lacking evidence to relate Haptoglobin genotype and malaria parasitemia can be due to the age of the study group as Hp^{2-2} has been associated with 30% reduction in malaria episodes but there was no protection in the first two years of life (Atkinson *et al*., 2007). Although this study doesn't show any evidence of influence of the Hp genotype on parasitemia prevalence, it gives a clue on the fate of infection. It is shown that the chance that malaria infection will result to complicated and severe malaria is greater in Hp^{2-2} than it is in Hp^{1-1}. This is inconsistent with another study that showed that using Hp^{1-1} as a reference, children with Hp^{2-2} exhibit a slightly increased risk of severe malaria (Bienzel *et al*., 2005).

The fact that protective effect of Haptoglobin against Malaria is age dependent (Atkinson *et al.*, 2007) may suggest that the Hp^{2-2} genotype may accelerate acquisition of immunity against malaria as has been suggested with sickle cell trait (Williams *et al*., 2005). It should be noted that the influence of Hp genotype is not on malaria alone. It has been found that the Hp^{1-1n}genotype is associated with significant protection against non-malarial febrile illnesses (Atkinson *et al*., 2007). Growing evidence suggests that Hp

plays an important role in host defence against infection. Hp^{1-1} is a ligand for the Mac-1 leukocyte integrin b2 (CD11b/CD18) receptor on monocytes, macrophages, and natural killer cells and it modulates leukocyte trafficking (El-Ghmati *et al*., 1996) and host response to bacterial lipopolysaccharides (Arredouani *et al*., 2005). Moreover, Hp suppresses lipopolysaccharide-induced release of TNF-a, IL-10, and IL-12 in vitro and protects the host against the deleterious effect of lipopolysaccharides in vivo (Arredouani *et al*., 2005). By binding to free Hb and reducing iron availability, Haptoglobin exerts a bacteriostatic effect on many organisms such as *Escherichia coli* (Eaton *et al*., 1982). Lower levels of available iron in the Hp^{1-1} phenotype compared to the Hp^{2-2} type (Asleh *et al.*, 2005) may thus limit bacterial growth. Clinical studies have shown that Hp^{2-2} is associated with increased mortality among patients with HIV infection (Delanghe *et al*., 1998) and tuberculosis (Kasvosve *et al*., 2005). A child below two years of age may not be benefiting the protective role of Hp2-2 genotype against malaria but the protective role of Hp^{1-1} against febrile diseases (Atkinson *et al*., 2007). This may explain the findings of this study that Hp genotype doesn't influence malaria parasite infections but may determine the fate of the infection in young children.

5.2 Haptoglobin level and Malaria

In this study it was found that the average plasma Haptoglobin level is 208.3 µg/ml in malaria negative samples and 159.3 µg/ml in malaria positive samples. A study by Freya *et al*. (2006) in Gabon found that a median Haptoglobin level in parasitemic children was 125 µg/ml. There were no ahaptoglobinea findings in this study probably because the ELISA technique used in quantification was sensitive enough to detect as low as 7.8 µg/ml. In malaria-endemic areas, low levels of Hp reflect recent parasitemia and malaria-induced haemolysis, as well as transmission intensity (Trape *et al*., 1988). In this

population, Hp levels were associated with Hp genotype but interacting with malaria parasitemia. The finding of most interest in this study was the interaction between Hp level, malaria parasitemia and Hp genotype. At negative parasitemia Hp levels are higher in Hp^{2-2} when compared to Hp^{1-1} and Hp^{1-2}. In contrast the Hp levels do not differ significantly between the genotypes in positive samples. However, the magnitude of change by the meaning of dropping Haptoglobin level was higher in Hp^{2-2}. Irrespective of the statistical significance, the Haptoglobin level is lower in Hp^{2-2} compared to the other genotypes.

The relationship between Hp levels and Hp phenotype has been reported in another study. (Langlois *et al*., 2006). Hp^{1-2} and Hp^{2-2} are polymeric, whereas Hp^{1-1} is a dimer (Dobryszycka *et al*., 2007). This structural form and the ability to form disulfide bridges to other Hp^{2} molecules, leading to a wide range of oligomers can therefore explain the fact that at negative malaria parasitemia, Haptoglobin level is higher in Hp^{2-2} when compared to Hp^{1-1} and Hp^{1-2}. Levels of Hp are reduced by both chronic, low level parasitemia and possibly malaria-associated immune complex destruction of infected erythrocytes, as well as clinical malaria (Trape *et al*., 1985; McGuire *et al*., 1996). Haemolysis secondary to malaria is the only significant cause of hypohaptoglobinaemia in African populations (Trape *et al*., 1985). It has been proposed that the distribution of haptoglobin levels may be useful in the evaluation of malaria control programs (Rougemont *et al*., 1988; Trape and Fribourg-Blanc, 1988; Sisay *et al*., 1992).

There are some other factors that can interact with malaria parasitemia to affect Haptoglobin level as it has been shown that in a malaria-endemic area of Papua New Guinea that individuals homozygous for α^{+}-

thalassemia had increased levels of Hp compared with heterozygotes when harbouring chronic parasitemia (Imrie *et al*., 2006).The binding of one Hb molecule to one molecule of a polymeric form of Hp and its subsequent clearance would result in a greater relative reduction in the total pool of Hp available compared with the clearance of one Hp^{1-1} dimer. It can be assumed that because of its larger size the Hp^{2-2} haemoglobin complex is taken up more efficiently by macrophages as compared with Hp^{1-2} or Hp^{1-1}. Moreover, Kristiansen *et al*. (2001) described a macrophage protein, CD163, a receptor that scavenges Hb/Hp complexes and that Hb/Hp^{2-2} complexes exhibited higher functional affinity for CD163 than do complexes of Hp1-1-Hb.

In summary, the present study found that Haptoglobin genotype doesn't influence susceptibility to malaria infection or development of the disease. It however showed that Haptoglobin genotype may influence the nature of the disease developed where Hp^{2-2} individuals express more of complicated and severe types of malaria as compared to the other studies. The findings of the relationship between the Haptoglobin genotype and frequency and susceptibility of malaria infection in this study unveiled the sensitivity of age factor in the relationship. The present study also showed that Haptoglobin genotype influenced the plasma Haptoglobin level both in parasitized and unparasitized patients. This was by showing that Hp^{2-2} level was higher in unparasitized individuals but lower in parasitized ones. Haptoglobin is associated with among other roles, taking care of the free Hemoglobin, a factor known to affect the body response to malaria infection. Haptoglobin accomplishes this task with the company of Heme Oxygenase, another polymorphic protein.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

Conclusively, from the present study the following information can be drawn;

i. The distribution of the Haptoglobin genotypes in the study group is in Hardy-Weinberg equilibrium.
ii. Haptoglobin genotypes do not influence susceptibility to Malaria infection
iii. Haptoglobin genotype influence the plasma Haptoglobin level where the level is higher in Hp^{2-2} compared to the other genotypes
iv. The extent of decrease in plasma Haptoglobin level is higher in Hp^{2-2} genotype than in the other genotypes

6.2 Recommendations

It is known that Haptoglobin is associated with among other roles, taking care of the free Hemoglobin, a factor known to affect the body response to malaria infection. Haptoglobin accomplishes this task with the company of Heme Oxygenase, another polymorphic protein. I recommend that an intensive study should be carried out to study how different combinations of different polymorphic forms of the two proteins express their influence in the susceptibility to Malaria infection and development of the disease.

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APPENDICES

Appendix 1; DNA Purification from Dried Blood Spots (QIAamp DNA Mini Kit)

Important point before starting

- All centrifugation steps are carried out at room temperature (15–25°C).

Things to do before starting

- Prepare an 85°C water bath for use in step 2, a 56°C water bath for use in step 3, and a 70°C water bath for use in step 4.
- Equilibrate Buffer AE or distilled water to room temperature for elution in step 10.
- Ensure that Buffer AW1 and Buffer AW2 have been prepared according to the instructions provided.
- If a precipitate has formed in Buffer AL or Buffer ATL, dissolve by incubating at 56°C.

Procedure

1. Place 3 punched-out circles from a dried blood spot into a 1.5 ml microcentrifuge tube and add 180µl of Buffer ATL.

Cut 3 mm (1/8 inch) diameter punches from a dried blood spot with a single-hole paper puncher.

2. Incubate at 85°C for 10 min. Briefly centrifuge to remove drops from inside the lid.

3. Add 20µl proteinase K stock solution. Mix by vortexing, and incubate at 56°C for 1h. Briefly centrifuge to remove drops from inside the lid.

Note: The addition of proteinase K is essential.

4. Add 200µl Buffer AL to the sample. Mix thoroughly by vortexing, and incubate at 70°C for 10 min. Briefly centrifuge to remove drops from inside the lid.

In order to ensure efficient lysis, it is essential that the sample and Buffer AL are mixed immediately and thoroughly.

Note: Do not add proteinase K directly to Buffer AL.

A white precipitate may form when Buffer AL is added to the sample. In most cases, the precipitate will dissolve during incubation. The precipitate does not interfere with the QIAamp procedure or with any subsequent application.

5. Add 200µl ethanol (96–100%) to the sample, and mix thoroughly by vortexing. Briefly centrifuge to remove drops from inside the lid.

It is essential that the sample and ethanol are mixed thoroughly.

6. Carefully apply the mixture from step 5 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.

Close each QIAamp Mini spin column in order to avoid aerosol formation during centrifugation.

7. Carefully open the QIAamp Mini spin column and add 500µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.

8. Carefully open the QIAamp Mini spin column and add 500µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.

9. **Recommended: Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.**

   This step helps to eliminate the chance of possible Buffer AW2 carryover.

10. **Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 150 µl Buffer AE or distilled water. Incubate at room temperature for 1 min, and then centrifuge at 6000 x g (8000 rpm) for 1 min.**

    Three punched-out circles (3 mm diameter) typically yield 150ng and 75ng of DNA from anticoagulated and untreated blood, respectively. If the yield from untreated blood is not sufficient, use 6 circles per prep instead of 3. The volume of the DNA eluate used in a PCR assay should not exceed 10%; for example, for a 50µl PCR, add no more than 5µl of eluate.

Appendix 2; Human Haptoglobin Quantitative ELISA Protocol

Buffer Preparation

1. Prepare the following buffers:

A. Coating Buffer, 0.05 M Carbonate-Bicarbonate, pH 9.6

B. Wash Solution, 0.05% Tween 20 in PBS pH 7.4

C. Blocking Solution, 1% Non Fat Dairy Milk in PBS

D. Sample/Conjugate Diluents, 50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0

E. Enzyme Substrate,

F. Stopping Solution, 2 M H_2SO_4 or other appropriate solution

Step-by-Step Method (Perform all steps at room temperature)

1. Coating with Capture Antibody

A. Dilute 55 µl of Capture Antibody in 11ml coating buffer to make a 1:200 dilution.

B. Add 100 µl per well.

C. Incubate coated plate for 60 minutes.

D. After incubation, aspirate the Capture Antibody solution from each well.

E. Wash each well with Wash Solution as follows:

i. Fill each well with Wash Solution

ii. Remove Wash Solution by aspiration

iii. Repeat for a total of 3 washes.

2. Blocking (Post-coat)

A. Add 200 µl of Blocking Solution to each well.

B. Incubate for 60 minutes.

C. After incubation, remove the Blocking Solution and wash each well three times as in Step 1.E.

3. Standards and Samples

A. Add 25 µl of Protein Calibrator to 975 µl sample diluent to prepare the highest concentration of Protein Calibrator solution (500 ng/ml). Prepare serial dilutions at a 1:2 (one part sample plus one part diluent) ratio to reach the lowest Protein Calibrator concentration (7.8 ng/ml).

B. Dilute the samples, based on the expected concentration of the analyte, to fit within the concentration range of the standards.

C. Transfer 100 µl of Protein Calibrator solutions and sample solutions to assigned wells.

D. Incubate plate for 60 minutes.

E. After incubation, remove samples and standards and wash each well 5 times as in Step 1.E.

4. Detection Antibody – Horseradish Peroxidase Conjugate

A. Dilute 1.4 µl of the HRP conjugate in 11 ml Conjugate diluent to make a 1:8000 dilution. (Adjustments in dilution may be needed depending on substrates used, incubation time, and other experimental conditions)

B. Transfer 100 µl to each well.

C. Incubate for 60 minutes.

D. After incubation, remove HRP Conjugate and wash each well 5 times as in Step 1.E.

5. Enzyme Substrate Reaction

A. Prepare the Substrate solution according to the manufacturer’s recommendation.

B. Transfer 100 µl of Substrate solution to each well

C. Incubate plate for 5-30 minutes.

D. To stop the TMB reaction, apply 100 µl of 2 M H_2SO_4 to each well. When using another substrate, follow the manufacturer's recommendations.

6. Plate Reading

Using a microtiter plate reader, read the plate at the wavelength that is appropriate for the substrate used (450 nm for TMB).