MOLECULAR CHARACTERISATION AND IMMUNO-EPIDEMIOLOGY OF *PLASMODIUM FALCIPARUM* INFECTION IN SCHOOL CHILDREN AT DODOWA

BY

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A THESIS SUBMITTED TO THE UNIVERSITY OF GHANA IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF PHILOSOPHY IN ZOOLOGY

DEPARTMENT OF ZOOLOGY
UNIVERSITY OF GHANA
LEGON, GHANA

MARCH, 1999

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This is to certify that this thesis has not been submitted for a degree to any other University. It is entirely my own work and all help has been duly acknowledged.

JOHNSON NYARKO BOAMPONG

DEDICATION

To my Uncle Kwaku Boampong and his family.

ACKNOWLEDGEMENT

I wish to express my sincere gratitude and deep appreciation to all individuals and institutions that, in diverse ways assisted me to successfully complete this work. I am especially thankful to Professor F.K. Nkrumah, Director of NMIMR for granting me permission to conduct research work at the institute and indeed the entire staff of the institute for their warm reception, kindness and help.

The work could certainly not have been completed without the patience, expert guidance, advice, constructive suggestions and criticisms from Drs. B.D. Akanmori, Head of Immunology Unit, J.A.L. Kurtzhals, visiting Research Fellow and M.D. Wilson, Head of Parasitology unit, all at the Noguchi Memorial Institute for Medical Research (NMIMR). Equally invaluable to the successful completion of the work was the experienced supervision of Dr Edoh at Zoology Department, University of Ghana-Legon. I am indeed very much indebted to them. I am also indebted to Messrs Ben Gyan, M. Ofori, M. Osei-Atweneboana, A. Hammond, Mrs Owusu, Ms A. Okyere, Mrs B. Ogoe, A. Richardson and A. Grace for helping me anytime I encountered problems.

The Danish International Development Agency (DANIDA) deserves to be thanked for providing funds through its ENRECA programme for this expensive work as part of the Accra-Copenhagen Research Link

I wish to thank my Uncle Mr Kwaku Boampong, my dearest wife Ms Georgina Thompson as well as friends for their encouragement, concern and companionship

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ABSTRACT

A longitudinal immuno-epidemiological study was conducted between April 1994 and August 1995 among Ghanaian children within the ages of 3-15 years, living in Dodowa, a stable malaria endemic area. A polymerase chain reaction (PCR) typing technique based on the amplification of polymorphic region of merozoite surface protein 1 (MSP1) of *Plasmodium falciparum* gene, was used to characterise parasites contained in blood spotted filter paper. The PCR assay detected as low as 2.2 parasites/μl of blood and revealed 35 MSP1 seasonally variable allelic forms in the children. Clinical malaria could not be attributed to any specific allelic type, and the acquisition of new allelic type was not necessarily associated with clinical malaria. Malaria episode was synchronised with significant increase in parasitaemia. Also anti-$MSP1_{19}$ antibodies were measured using Enzyme-linked immunosorbent assay (ELISA) from blood samples collected from 27 of these children before and after malaria attack. Antibody responses to the C-terminal of 19kDa fragment of *Plasmodium falciparum* before malaria attack showed 14 children as positive and 13 negative. Nine of the children were within the ages of 3-4 years whereas eighteen were 5 years and above. Four children out of the nine aged 3-4 years were negative before malaria attack but three showed negative to positive sero-conversion. In contrast 2 out of 9 children who were positive before malaria attack showed positive to negative sero-conversion.

CHAPTER ONE

INTRODUCTION

Malaria is a mosquito-borne protozoal disease caused by species of the genus *Plasmodium*. It is characterised by acute febrile illness which may be expressed as periodic paroxysms occurring every 48 or 72 hours with afebrile and relatively asymptomatic intervals and the tendency to recrudesce or relapse over a period of months to many years (Gilles and Warrel, 1993). Other major symptoms of malaria are rigors/chills, vomiting, convulsion, headache, drowsiness and muscle-skeletal pain. It is a significant cause of abortion, stillbirth, child mortality, low birth weight, death in pregnant women, impaired growth in children and loss of productive activity in adults (TDR, 1987). Malaria is widespread and endemic throughout many parts of the world, especially the tropics and subtropical regions. It is estimated to kill between 1.5 and 2.7 million people every year. Another 300 to 500 million people have the disease and one third of all mankind live in zones where they are exposed to the risk of infection (Butler, 1997). The vast majority of malaria deaths occur among young children in Africa, especially in remote rural areas with poor access to health services. Outside tropical Africa, deaths from malaria occur principally among non-immune people who become infected with *P. falciparum* in areas where appropriate diagnosis and treatment are not available (Theander, 1991). The enormous number of lives and labour lost together with the cost of treatment of patients exerts a negative impact on development and makes malaria a major economic burden (TDR, 1993). Chemotherapy, hitherto the most

effective control method has become less effective as a result of the development of drug resistant malaria parasites, in addition to insecticide resistance in the anopheline vectors (Gilles and Warrel, 1993). This has led to a focus on anti-malaria vaccine development as an additional tool for the control of the disease.

The four parasite species that infect man are *P. vivax, P. malariae, P. ovale* and *P falciparum*, the latter being by far the most lethal. *Plasmodium vivax* has the widest geographical range. It is prevalent in the tropic and subtropical regions and is also found in some temperate zones. *Plasmodium malariae* is patchily distributed over tropical Africa, Eastern Asia, Oceania and Amazon area. *Plasmodium falciparum* is the commonest malaria parasite in tropics and subtropics and is predominant over the same range as *P. malariae*. It is mainly found in East and West Africa, Guyana and parts of India. *Plasmodium ovale* is mainly found in tropical regions such as West Pacific regions, southern China, Burma and South East Asia (WHO, 1993).

Traditional malaria diagnosis is based on the standard microscopical examination of Giemsa stained blood films to identify the parasite. Differential diagnosis is made possible by the morphology of the parasites but accurate species differentiation may be difficult especially in patients with very low circulating parasitaemia and mixed infection of *P. ovale* and *P. vivax* (Milne *et al.*, 1994). In malaria endemic areas, varying proportions of the population continuously carry low grade and asymptomatic parasitaemia but periodically show clinical episode associated with sharp rise in parasite densities (Contamin *et al.*, 1996).

The development of the PCR, which is the enzymatic amplification of parasite specific DNA sequences (Saiki *et al.*, 1988) has made possible the detection of low parasitaemia in infected humans (Snounou *et al.*, 1993).

Increased molecular characterisation of a number of functional *P. falciparum* genes and gene families, has placed at the disposable of researchers a suitable means of typing the parasite. One such source of variation suitable for typing *P falciparum* strains is the gene that codes for the merozoite surface protein MSP1.

Experimental infections in humans have shown that the immunity raised to one strain of *P falciparum* is largely inefficient against challenge with heterologous strains (Jeffery, 1996). This raises the question whether only increased populations of parasitaemia or just a new virulent strain of the parasite irrespective of the density that causes clinical malaria.

The erythrocytic stage of *P falciparum* is responsible for the clinical malaria and as such antigen associated with this stage may be of importance in the development of protective immunity of the disease (Gilles and Warrel, 1993). One such well characterised antigen is the *P falciparum* merozoite surface protein 1 (PfMSP1) that remains attached to the merozoite during erythrocytic invasion and is also expressed by the parasite during early ring stages (Holder *et al.*, 1987). Antibodies against this fragment may block merozoite invasion of erythrocytes and also inhibit multiplication inside the erythrocytes (Blackman and Holder, 1992). The PfMSP1 gene has been sequenced and it is now known to consist of 17

blocks that is either highly conserved, semiconserved or variable. The polymorphic region at the 5' end of the gene in block 2 varies extensively in number and in sequence detail of repeats. This provides ideal basis to discriminate strains of the parasite between isolates (Tanabe *et al*, 1987). The polymerase chain reaction is now used to distinguish *P. falciparum* strains using primer sequences that flank the polymorphic region of MSP1 gene (Conway and McBride, 1991; Ranford-Cartwright *et al*, 1993).

1.1 Rationale and objectives

The interaction between the human host and infecting malaria parasites is prerequisite to an understanding of the mechanisms underlying the pathogenesis and acquisition of immunity against malaria infection. Studies have shown that immunity to malaria parasites has marked strain specific component. Different parasite strains have also been shown to vary in their clinical and pathogenic properties as well as their susceptibility to various drugs (Snewin *et al*., 1991; Babiker *et al*., 1995; Conway *et al*., 1991). The acquisition of immunity to malaria is not rapid but rather a slow process (Theander, 1991) and may be due to lack of exposure to a wide range of parasite strains.

Another possible phenomenon that might influence immune status is the carriage of low-grade parasitaemia in the blood of individuals, which may induce clinical protection. This has therefore necessitated an investigation into clinical protection from malaria and to provide useful information for understanding of epidemiology of the disease condition.

1.2 Specific objectives

1. To determine the sensitivity of the polymerase chain reaction (PCR) assay for parasite detection in blood collected on filter paper.
2. To use PCR to discriminate between *P. falciparum* strains and to test the hypothesis that a shift from asymptomatic to symptomatic clinical malaria is caused by a new strain.
3. To investigate a possible correlation between antibody responses to $MSP1_{19}$ and protection from clinical malaria.

CHAPTER TWO

LITERATURE REVIEW

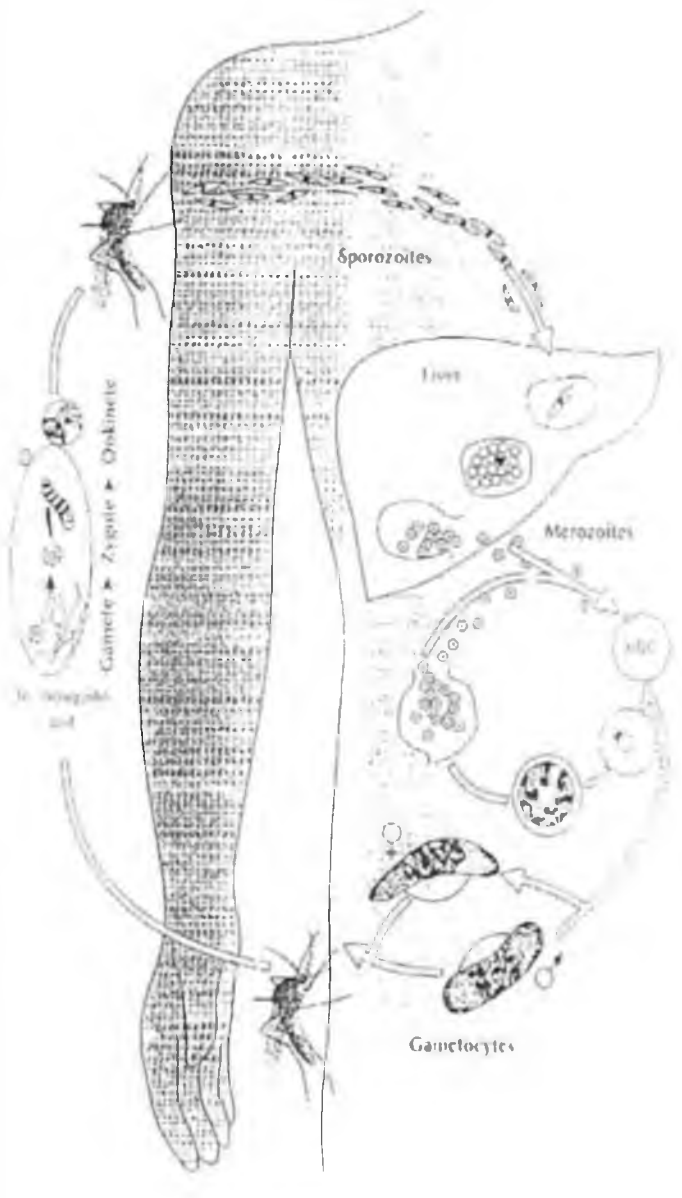
2.1 Classification of *Plasmodium* species

Plasmodium species are unicellular, eukaryotic and parasitic organisms belonging to the phylum Protozoa, subphylum Apicomplexa, class Sporozoa, subclass Coccidia, Order Coccidiida, suborder Haemosporina, family Plasmodiidae and genus *Plasmodium* and *Laverania* (Manson-Bahr and Apted, 1987). There are four species that infect man namely, *P. vivax*, *P. malariae*, *P. ovale* and *P. falciparum*. However this zoological classification of *Plasmodium* species is not universally accepted especially with regards to the differences of opinion in the taxonomic position of the parasite causing *falciparum* malaria. Some authors maintain that it belongs to a separate genus *Laverania* (Gilles and Warrel, 1993) as a result of its crescentic shape and lengthy development. Others are of the opinion that the rejection of the familiar name *Plasmodium falciparum* might be confusing and since the use of this well known name is still taxonomically permissible, it should be retained (Gilles and Warrel, 1993).

2.2 The life cycle of *Plasmodium* species

The life cycle of all *Plasmodium* species infecting humans is complex and largely similar. It consists of an endogenous asexual phase (schizogony) in the human host and exogenous sexual phase (sporogony) in the female anopheline mosquito as illustrated in Figure 1.

Figure 1. Schematic representation of the life cycle of malaria parasites in human and mosquito host (reproduced from Good *et al.*, 1988)



2.2.1 Schizogony

Viable sporozoites are inoculated into the bloodstream of humans by an infected female *Anopheles* mosquito when it is taking a blood meal. Within thirty minutes the sporozoites disappear from circulation. Although phagocytes destroy most sporozoites a few enter the parenchymal cells (hepatocytes) of the liver directly or via the Kuffer cells (Gilles and Warrel, 1993). The asexual phase (schizogony) in humans involves two stages; the exo- and erythrocytic stages.

2.2.1.1 Exo-Erythrocytic stages

The invasion of hepatocytes by sporozoites leads to development and multiplication (schizogony). A fully matured exo-erythrocytic schizont, which contains 10,000-30,000 merozoites, ruptures and releases its contents into the bloodstream within the period of one to three weeks after sporozoite inoculation. Some merozoites of *P. vivax* and *P. ovale* remain in hepatocytes, forming hypnozoites, which may repeat the process of schizogony thus causing relapses weeks to years later. *Plasmodium falciparum* and *P. malariae* however do not have a hypnozoite stage (Gilles and Warrel, 1993).

2.2.1.2 Erythrocytic stage

Most of the liberated merozoites invade the erythrocytes present in the sinusoids of the liver, but some are phagocytosed. The rest enter into circulation and invade other erythrocytes as described by Holder *et al.* (1987); Blackman and Holder (1992). Once inside the erythrocytes they form trophozoites which initially appear as characteristic 'rings'. The trophozoites then mature and undergo another

asexual multiplication to produce mature schizonts, which contains 2000 new merozoites. The erythrocytes rupture releasing the merozoites which then invade other erythrocytes. This erythrocytic cycle of schizogony is repeated over and over again, leading to a progressive increase of parasitaemia until the process is slowed down by the immune response (Gilles and Warrel, 1993). *Plasmodium falciparum* like *P. vivax* and *P. ovale* complete the erythrocytic cycle within 48 hours, whereas the cycle of *P malariae* takes 72 hours. This tends to be synchronous, producing periodic fevers with successive releases of the merozoites. Parasite levels in the peripheral circulation are known to vary during the erythrocytic cycle. In *P. falciparum* only trophozoites, which are younger than 18-21 hours old, are seen in peripheral circulation. The reason for this is sequestration of the maturing stages in the inflamed endothelia of capillaries and vessels. After several cycles the end of the schizogonic periodicity, sexually differentiated forms, gametocytes emerge as illustrated in Figure 1. In synchronous infections of some species of *Plasmodium*, gametocytes mature at night indicating an adaptation of the parasite to nocturnal feeding habits of female anopheline mosquitoes (Gilles and Warrel, 1993).

Clinical malaria follows the pattern of distribution of intraerythrocytic stages in humans and presents a broad range of symptoms. These include repeated generalised convulsions, normocytic anaemia with haemocrit < 15%, impairment of consciousness or unrousable coma, jaundice, hypoglycaemia, weakness, fever, rigors/chills, headache and fever.

2.2.3 Sporogony

When a female *Anopheles* mosquito ingests the blood of a human host, which has circulating malaria parasites, the asexual parasites are digested together with the blood cells, but gametocytes undergo further development. The male gametocytes (microgametocytes) exflagellate mates with female gametocytes (macrogametocytes) to form zygotes in the mosquito midgut. The zygote then transforms into a motile ookinete, which penetrates the intestinal walls of the midgut where it lodges to form the oocyst. It then undergoes sporogony and bursts after maturation releasing thousands of spindle-shaped sporozoites into the haemocoele. The sporozoites migrate to the salivary glands from which they are inoculated into humans to complete the cycle

2.3 Malaria in Ghana

Malaria still poses a major public health problem in Ghana and tops the ten most common causes of morbidity reported by Centre for Health Information Management (CHIM) from 1990-1994 (MOH, 1994). It is also the commonest cause of out-patient disease (OPD) conditions. All Ghanaians are at risk of being infected but it mostly affects children under 5 years, pregnant women and non-immune visitors. It is the disease with high mortality in late infancy and early childhood (MOH 1987).The disease occurs throughout the year but more prevalent during and after the rains. The disease prevalence varies between zones being highest in transitional savannah zones (Afari *et al*., 1992).

Plasmodium falciparum is the most common parasite in Ghana (Coulbourne and Wright, 1955). It is responsible for more than 90% of malaria cases, followed by *P. malariae* which can sometimes cause more than 10% of total infections in the dry seasons, especially in the coastal savannah area. *Plasmodium ovale*, which is rare in Ghana, has a patchy distribution and accounts for 0-15% of infections (MOH, 1994). Mixed infections of *P. falciparum* and *P. malariae* are also generally high in dry seasons (Afari *et al.*, 1992). *Plasmodium vivax*, which hitherto has not been recorded in Ghana, was recently identified in blood sample from an American who visited Ghana (Kain *et al.*, 1991). Binka *et al.* (1994) have reported that *Plasmodium falciparum* constitutes 1.4% of *Plasmodium* infections at Kassena-Nankani District of Upper East Region. Malaria transmission also varies from region to region with intense transmission rates being recorded during the season when mosquito vectors are abundant. However, even within this pattern, different transmission rates exist due to a combination of factors, mainly environmental conditions that influence the distribution of vectors and thus the disease.

The distribution of malaria within human populations in Ghana is closely linked to site-specific characteristics of vector populations. These include abundance, susceptibility to infection, longevity and their contact with human. This is complemented by human habits that actively promote man-mosquito contact (Afari *et al.* 1994).

Studies conducted in Ghana have revealed that the principal vectors of malaria are

member species of the *An. gambiae* complex and *An. funestus* (Afari *et al.*, 1992). There are six recognised species of the *An. gambiae* complex, namely, *An. gambiae sensu strictu*, *An. arabiensis*, *An. melas*, *An. quadriannulas*, *An. merus* and *An. bwambae*. Appawu *et al.* (1994) have reported only three of the six members of the *An. gambiae* complex; *An. gambiae sensu strictu*, *An. arabiensis* and *An. melas* occur in varied eco-epidemiological zones in Ghana. *Anopheles arabiensis* is found in the arid north of the country and in lower densities in the coastal mangrove and swampy areas where *An. gambiae ss* predominates. *Anopheles melas* plays the predominant role in malaria transmission in the coastal areas during dry season but *An. gambiae ss* assumes this position during the rains.

The principal vector populations have the ability to maintain the transmission of different *Plasmodium* parasites and are able to survive in the rapidly increasing environmental changes such as creation of stagnant water bodies in the country, thus making strategic control measures ineffective.

2.5 Genetics of *Plasmodium falciparum*

Chromosomes of intraerythrocytic stages of *P. falciparum* have been determined in relation to the number of kinetochores by electron microscopy and by pulse field gel electrophoresis (PFGE) of intact chromosomes (Prensier and Slomianny, 1986). It has been revealed that there are 14 chromosomes that range in size from 600kb to 3.5Mb. The chromosomes undergo re-assortment and crossing-over during cross fertilisation between genetically different strains which co-infect the mosquito, resulting in high frequencies of recombinant progeny (Conway and

McBride, 1991; Lanzer *et al.*, 1994).

All the chromosomes except two have been assigned 25 genetic markers (Kemp *et al.*, 1987). The genes of most *P. falciparum* antigens that have been identified so far, are located at subtelomeric regions of chromosomes where they are prone to recombination and deletion (Lanzer *et al.*, 1994). Most of these genes contain blocks of tandemly repeated coding sequences (Kemp *et al.*, 1987). It has been speculated that the major contributor to chromosome size polymorphism in malaria parasites is the difference in the amount of repetitive DNA present in the genome of an individual parasite. Lack of strong selective pressure may permit dramatic fluctuation in the abundance of repetitive DNA with consequential chromosome size polymorphism between parasites (Conway and McBride, 1991). Size polymorphisms in *P. falciparum* do not involve interchromosomal exchange of large segments of DNA. In contrast, *Trypanosoma brucei* genes for variant surface glycoprotein (VSG) of the surface coat of the parasite undergo rearrangement that regulate their expression and transpose large segments of DNA from one chromosome to the other. This in *T. brucei* generates marked chromosome size differences thereby displaying remarkable heterogeneity (Van der Ploeg and Cornelissen, 1984). The diversity of *P. falciparum* has been well demonstrated in variant forms of antigens, proteins, enzymes, resistance/susceptibility to antimalarial drugs and sequences of many genes (Wataya *et al.*, 1993; McBride *et al.*, 1982; Fenton *et al.*, 1985; Sanderson *et al.*, 1981; Tirasophon *et al.*, 1994; Peters, 1987 and Lockyer and Schwarz, 1987). There is also evidence that some variants of these characters occur at different

frequencies in different geographical areas (Walliker *et al.*, 1987), which can be discriminated using one or more genetic marker(s) that represent(s) allelic forms of each respective gene. One such antigen, which exhibits allelic polymorphism and also varies in frequencies in different geographical areas, is the *P. falciparum* merozoite surface protein 1 (PfMSP1). The antigen MSP1, also identical with the precursor to the major merozoite surface antigen (PMMSA/gp195) and merozoite surface protein 1 (MSA-1) (Peterson *et al*, 1988; McBride *et al.*, 1985; Holder and Freeman, 1982), is encoded by a single locus gene located on chromosome 9 (Kemp *et al.*, 1987; Walker-Jonah *et al.*, 1992; Anders *et al.*, 1993).

2.6. Merozoite surface protein 1 (MSP1)

The merozoite surface is made up of multiple proteins seemingly 'fuzzy' or amorphous but organised 'spikes' of 20-25 nm projections (Galinski and Barnwell, 1996). The MSP1 which is major surface antigen of *P. falciparum* merozoites is a glycoprotein of 185-205 kDa molecular weight (Holder and Freeman, 1982; Holder *et al.*, 1987). It is derived from a high molecular weight precursor molecule identical with gp 190, gp 195 and PMMSA, synthesised during schizogony and expressed on the surface of intraerythrocytic parasites (Holder and Freeman, 1984).

Plasmodium falciparum MSP1 (PfMSP1) is proteolytically processed into fragments of 83, 42, 38, 28-30 and 19 KD at the end of schizogony, just prior to the release of merozoites and can be found on the surfaces of mature merozoites. However only C -terminal fragment of 19 KDa ($MSP1_{19}$) remains on the

merozoite surface during invasion of a new erythrocyte (Holder *et al.*, 1987; Holder and Freeman 1984; Blackman *et al.*, 1990; Blackman and Holder, 1992). When the parasite invaginates into a new erythrocyte, it forms parasitophorous vacuoles that separate it from the surrounding cytoplasm.

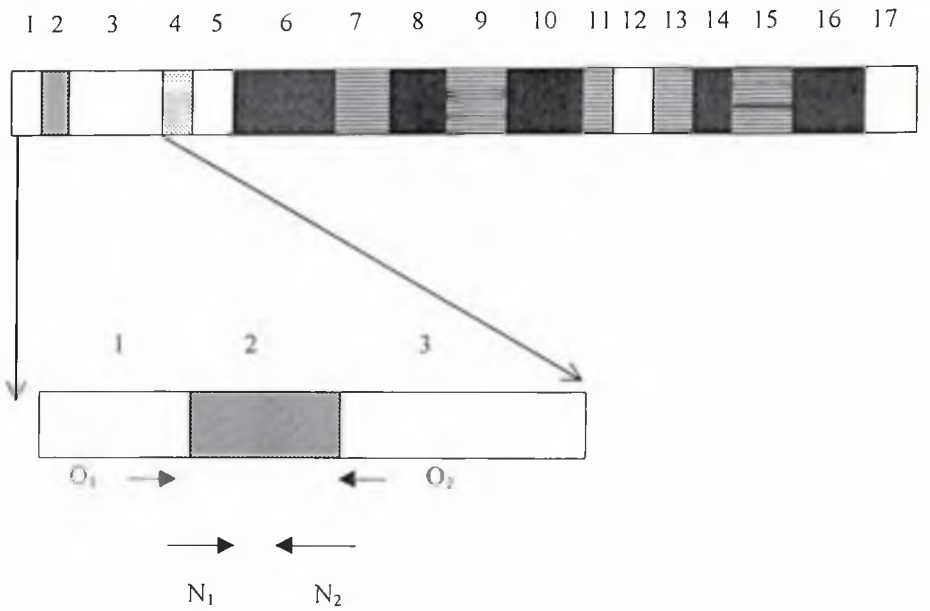
The role of MSP1 is to recognise and aid adherence to the membranes of erythrocytes (Howard and Pasloske, 1993). The proteins produced by MSP1 gene are also polymorphic in natural populations of *P. falciparum*. The reactivities of MSP1 strain specific monoclonal antibodies have shown a large number of this molecule, each being encoded by different allele of the MSP1 gene (Holder and Freeman, 1982).

2.6.1 The structure of *P. falciparum* MSP1 gene

The PfMSP1 gene has been sequenced and shown to consist of 17 blocks that are either highly conserved, semi-conserved or variable (Figure 2). In five of these blocks (1, 3, 5, 12, and 17) the DNA sequences are conserved in all isolates. Seven regions of the gene show extensive polymorphism (blocks 2, 4, 6, 8, 10, 14, and 16), whilst the remaining parts (block 7, 9, 11, 13 and 15) appear to consist of conserved sequences with patches of non-homologous sequence (Tanabe *et al.*, 1987). Surprisingly, the variable and semi-conserved regions (ie 2, 4, 6, 7, 8, 9, 10, 11, 13, 14, 15, 16) exist only in two dimorphic forms. The only exception to this is the polymorphic tripeptide region at the 5' end of the gene in block 2. The allele K1 and MAD20 are considered as the allelic prototypes from which other allelic forms probably have been generated by intragenic recombination at the 5'

end of the gene (Tanabe *et al.*, 1987). Using PCR analysis Certa *et al.* (1987) showed that a third polymorphic form of the MSP1 gene, called R033 lacks the N-terminal tripeptide repeats. This study examined the polymorphic region (block 2) using two sets of primers (outer and inner primers)

Figure 2: A simplified scheme illustrating the MSP1 gene of *P. falciparum*.



Key

A= Conserved block B = Repetitive block 2 C = Semi-conserved block

D and E = 'Dimorphic' non-repetitive blocks

The MSP1 gene is divided into blocks on the basis of polymorphism. Block 2 contains highly polymorphic tripeptide repeats. The positions of the primers used for PCR in this study are shown by arrows (see above). The outer primers are denoted O_1 and O_2 and the nested primers are denoted N_1 and N_2 (modified from Ranford-Cartwright *et al.*, 1993)

2.7 Host-Parasite interaction

In malaria endemic areas not all individuals are equally susceptible to infection by malaria parasites, and even if an infection is established, the associated morbidity is dependent on a number of intrinsic factors. These factors collectively define the resistance or immunity to malaria. The human immune response to infection with the malaria parasites is complex involving natural or innate immunity and acquired immunity (reviewed by Gupta *et al.*, 1994a).

2.7.1 Natural or innate immunity

Innate immunity is independent of previous exposure since it confers no adaptive immunological memory in response to infection. A good example is the protection against vivax malaria afforded to Duffy-negative individuals, who lack the erythrocyte surface antigen used by *P. vivax* merozoite during their invasion of red cells (Gilles and Warrel, 1993). The geographical distribution of malaria and certain red cell disorders such as thalassaemia and haemoglobinopathies suggest that the deleterious anomalies may be counter-balanced by a relative protection against malaria (reviewed by Hviid, 1995).

2.7.2 Acquired immunity

The acquisition of immunity is slow, requiring many years of exposure to the parasite, and numerous disease episodes. The acquired immunity is practically not complete, and residents of malaria endemic areas continue to experience sporadic

episodes of clinical disease throughout life. However, the incidence and density of malaria parasitaemia decline with age. Where age-dependent acquisition of anti-malarial immunity does occur, the slow development and fragility seemingly reflects the largely strain- and stage -specific nature of induced immune response (reviewed by Mercereau-Puijalon *et al.*, 1991a; Good *et al.*, 1988). It is believed that immunosupression during acute episodes of malaria may interfere with development of adequate protective immunity (reviewed by Hviid, 1995).

The effector cells (natural killing cells (NK), macrophages and granulocytes) participate in immunity through lysis and phagocytosis of parasitized erythrocytes as the first line of action against infection, prior to antigen specific sensitisation (Taverne *et al.*, 1986). The specific immune defense mechanism against malaria parasites involves the participation of T and B cells. The T-cells occur as (1) helper cells for antibody production, (2) the activation of non-specific effector cells, such as macrophages and granulocytes, (3) the producer of substances toxic to the parasites and (4) cytotoxicity cells. B-cell responses in human malaria involve the synthesis of antiplasmodial antibodies with the immunoglobulin types IgA and IgM responses tending to be transient while IgG responses are more persistent. Immunoglobulin G is progressively raised in individuals infected with malaria parasites (Collins *et al.*, 1971). The rate of IgG synthesis is three times as high in unprotected individuals than protected ones, all living in an endemic area. It is also seven times higher in non-immune people (reviewed by Theander, 1991). Both antibody-independent and antibody-dependent mechanisms appear to be involved in acquired immunity to malaria. The antibody independent host

defense mechanism against malaria infection involves reticuloendothelial cell hyperplasia with various leucocytic responses (Sheagren *et al.*, 1970).

The immune responses providing the selection pressure for variation in MSP1 would be even more compelling if the variable rather than conserved regions of the molecule were shown to be preferred location of T- or B- cells epitopes (Anders and Smythe, 1989).

The interactions between different parasites simultaneously infecting the same individual may result in antagonism between the species significantly changing the course of the infection and its potential to produce disease (Riche, 1988; Snounou *et al.*, 1992). Antibody production during malaria parasite infection is age dependent in endemic population and children develop antibodies against variant epitopes whereas adults develop antibodies against less immunogenic but conserved epitopes. In addition antibodies against immunodominant epitopes often cross-react with other repeated epitopes, either within the same molecule or on other parasite proteins (Theander, 1991, Anders, 1986). Cross-reaction interferes with the maturation of high affinity antibody directed against the parasite by causing an abnormally high proportion of somatically mutated B-cells to be preserved during clonal expansion. The cross-reacting epitopes could interfere with the absorption of antibodies on antigens that are not essential for the survival of the parasite, thus simply diverting immunological response from more important epitopes (Blackman *et al.*, 1990; Shai *et al.*, 1995).

Antibodies against the C-terminal fragment, $MSP1_{19}$ may block merozoite invasion of erythrocytes, and also inhibit parasite multiplication inside the erythrocytes (Holder and Freeman, 1984; Blackman *et al.*, 1990).

In some cases, vaccinated animals produce invasion-blocking antibody and are protected from challenge, whereas in other cases, protection develops without production of blocking antibodies (Wyler and Pasvol, 1986). The experimental evidence for protective effect of anti-$MSP1_{19}$ antibody is thus inconsistent as Dodoo *et al.* (1999) found no significant association between the prevalence or levels of anti-$MSP1_{19}$ antibody with protection from malaria.

2.8 Immuno-Epidemiology of *P. falciparum* malaria

The severity of a *P. falciparum* infection depends on the complex interplay of the immune status, genetic background, and possibly on parasite virulence factors such as invasive efficiency, intraerythrocytic maturation and replication rate (Contamin *et al.*, 1996). There is still a debate on the hypothesis that some *P. falciparum* strains might be more virulent than others (Gupta *et al.*, 1994b).

Experimental infections in humans have indeed indicated that some strains consistently induced more severe infections than others. It has also been shown that the onset of symptoms in chronically infected previously asymptomatic individual may be correlated with introduction of a new parasite strain, differing from that in the original infection (James *et al.*, 1932; Contamin *et al.*, 1996).

It has also been proposed that during infection with a single parasite strain all the

variants are expressed within few cycles and presumably antibodies against all or most of these variants are induced during primary attack. If the immune response against any given variant declines rapidly following its elimination, that variant may reappear later due to switching in other not yet eliminated variants (Staalsoe and Hviid, 1998). Jeffery (1996) observed that in humans immunity raised to a particular strain of *P. falciparum* is largely inefficient against challenge of heterologous strains. Day and Marsh (1991) showed that malaria is characterised by clinical disease and acquisition of protection is slow since a long period is required to achieve exposure to a large repertoire of serotypes.

It has been observed that in the absence of transmission by mosquitoes in low endemic regions, malaria attack results from a single infective bite and no major allelic changes occurs, and that the parasites are propagated for a long time without major modification at loci used as markers. Intense transmission period however is marked by considerably changed genotypes and that rapid turnover may reflect frequent renewal of parasite populations resulting from sporozoite inoculation (Daubersies *et al.* 1996; Paul and Day, 1998). There is ample evidence that malaria parasites are polymorphic for a large number of genetic diversity of local parasite populations. The number of allelic types to which people are actually exposed is essentially unknown and little is known about the circulating strains in a restricted geographic area (Conway and McBride, 1992; Forsyth *et al*., 1989). It is believed that immunity in African children is strain specific and that clinical attacks are caused by new strains. The elucidation of the contributory role that specific strains play in acquisition of clinical protection

against malaria should be sought

Although microscopic examination of blood films is a cheap and reliable method to establish malaria infection, detailed studies of the parasite necessitate the use of other methods to augument its sensitivity. There are indirect fluorescent antibody test (IFAT), the indirect haemagglutination (IHA) test, immuno-precipitation techniques (double gel diffusion test), the enzyme-linked immumsorbent assay (ELISA) and polymerase chain reaction (Gilles and Warrel, 1993). To investigate *P. falciparum* strains with respect to their antigenic variation and the immune response of humans PCR and ELISA were respectively chosen in the present study.

2.9 Polymerase chain reaction (PCR)

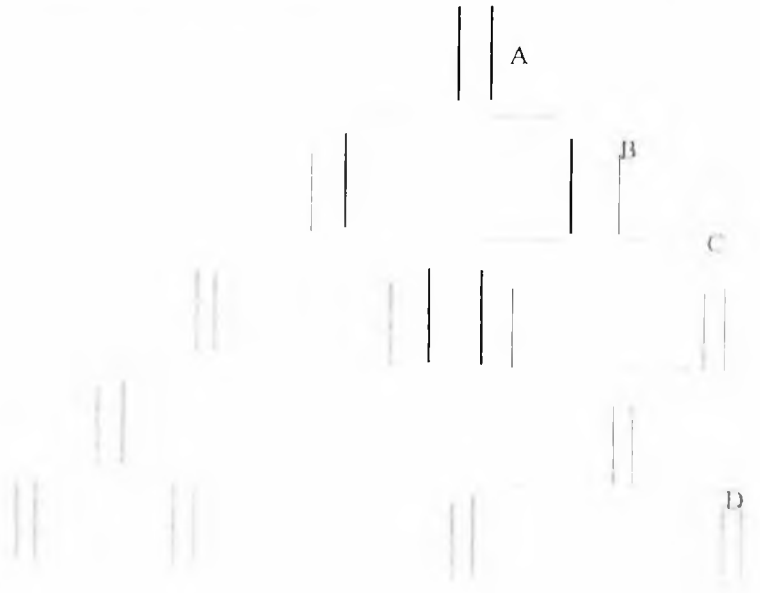
At present infected-cell agglutination test (Brown and Brown, 1965) and variant forms of enzymes, antigens, proteins, gene sequences and drug susceptibility have been used to characterise *P. falciparum* (Creasy *et al.*, 1990). Isoenzyme typing for example (Babiker *et al.*, 1991; Carter and McGregor, 1973) requires substantial amount of parasites, restricting the analysis of samples with low levels of parasitaemia. Monoclonal antibody typing requires parasites at particular stage of development, necessitating short-term maturation of blood stage parasite (McBride *et al.*, 1984; Conway *et al.*, 1991; Conway and McBride, 1992). It is neither practical nor representative to limit epidemiological studies to cases where sufficient parasite material can be obtained directly from patients.

The PCR has become a major diagnostic and research technique. The superior sensitivity and accuracy of the PCR assay over microscopical diagnosis has been established (Snounou *et al*., 1993). It has the major advantage of eliminating the need for *in vitro* manipulation of parasites because DNA from circulating ring stage can be used to analyze a large number of genetic loci, including those expressed at different stages or in mosquito vector. Moreover PCR generates the material to be typed, instead of consuming it, and once a gene fragment has been amplified, analysis of the fragment can be performed by various methods (Contamin *et al*., 1995). However PCR is expensive and hardly used for routine diagnosis. There is also the possibility of contamination resulting from handling of templates and PCR products.

The polymerase chain reaction is a rapid procedure for *in vitro* exponential amplification of a specific target DNA sequence mediated by enzymes (Saiki *et al*., 1988). It involves two oligonucleotide primers that flank the DNA fragment to be amplified. There are repeated cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences, and extension of the annealed primers with DNA polymerase as illustrated in Figure 3. The primers hybridize to opposite strands of the target sequence and are orientated so that DNA synthesis by the polymerase enzyme proceeds across the region between the primers. The extension products themselves are also complementary templates and are capable of binding primers. Successive cycles of amplification essentially doubles the amount of the DNA synthesized in the previous cycle. The result is the exponential accumulation of the specific target fragment, which then can be

visualised by gel electrophoresis (Saiki, 1990). The high sensitivity, specificity and yield of PCR with the thermostable *Taq* DNA polymerase make it an ideal method for the isolation of a particular genomic fragment (Saiki, 1990) The analysis of PCR amplified DNA products encoding polymorphic protein of *P falciparum* allowed the determination not only of species, but also subspecies or strains (Paul *et al.*, 1995).

Figure 3. Schematic representation of PCR amplification Process.



—— Original DNA

—— PCR primer

—— New DNA

A: DNA + primers + dNTP + DNA polymerase

B: Denature and synthesize

C: Denature and synthesize

D: Denature and synthesize

DNA to be amplified is denatured by heating the sample. In the presence of DNA polymerase and excess deoxynucleotide triphosphates, oligonucleotides that hybridize specifically to the target sequence can prime new DNA synthesis. Depending on the number of cycles many millions of discrete DNA fragments can be generated

2.10 Analysis of PCR products by electrophoresis

Electrophoresis is a technique which ensures that charged molecules in solution, usually proteins and nucleic acids migrate in response to an electrical field (Patel, 1994). The molecules migrate based on the strength of the electric field, the net charge, size and shape of the molecules, the ionic strength, viscosity and temperature of the medium in which the molecules are moving. In general the rate at which fragments move is a function of their sizes or lengths and charges that they bear, with small fragments moving much faster than large ones. The lengths of PCR products are estimated by comparison with molecular weight markers of known sizes on an agarose gel. In principle the migratory rates of molecules on agarose gels are inversely proportional to the logarithm of the molecular weight (Helling *et al.*, 1974) and is expressed as D = a - b (log M), where D is the distance travelled by the DNA fragments, M the molecular weight of DNA and, a and b are constants. Electrophoresis is usually carried out in gels and the most commonly used support matrices in which the sample is run are agarose and polyacrylamide.

2.10.1 Agarose gel electrophoresis

Agarose gel electrophoresis is a tool in the investigation and characterisation of DNA molecules. It is rapid, precise and inexpensive and requires only small amounts of DNA (Jeyaseelan *et al.*, 1987). The gel is made by heating the granular material in the appropriate electrolyte buffer, casting the gels and cooling. The

resolving power of these gels depends on the concentration of the dissolved agarose. Less concentrated gels are used for very large DNA molecules and the concentrated gels for lower molecular weight size DNA.

DNA is colourless therefore a DNA binding visible substance, Ethidium-bromide is added to the gel. It makes ultraviolet light chemically luminate into visible light when it is bound to nucleic acids. This electrophoresis is usually performed using Tris acetate EDTA (TAE) buffer but Tris borate EDTA (TBE) can also be used

2.10.2 Polyacrylamide gel electrophoresis (PAGE)

The rationale for polyacrylamide gel electrophoresis is identical to that of agarose. However , it has a resolution power for defining DNA fragments of different lengths is greater and within a size range where agarose gel is less informative (Dawson *et al.*, 1996). A polyacrylamide gel is better for separating small fragments of DNA. It invariably have the following three major advantages over agarose gels. The resolving power is so great that it can separate molecules of DNA whose lengths differ by little as 0.2% (i.e. 1 bp in 500bp). It can accommodate much larger quantities of DNA than agarose gels. DNA recovered from polyacrylamide gels is extremely pure (Sambrook *et al.* 1989).

There are two common types of polyacrylamide gels which are often used i.e. non-denaturing and denaturing.

2.10.3 Discontinuous non-denaturing polyacrylamide gel

Most double stranded DNA migrates through non-denaturing polyacrylamide gels

at a rate that is approximately inversely proportional to the log $_{10}$ of their size. However, their base composition and sequence also affect their electrophoretic mobility, so that DNAs of exactly the same size can differ in mobility up to 10%. This effect is believed to be caused by kinks that form at specific points of double stranded DNA. Since it is impossible to know whether or not the migration of an unknown DNA is anomalous, electrophoresis through non-denaturing polyacrylamide gels cannot be used to determine the size of double stranded DNA (Sambrook *et al.*, 1989). It is therefore used to widely separate the bands, which hitherto have been close on agarose gel.

2.11 Enzyme-linked immunosorbent assay (ELISA)

Enzyme immunoassay was first described by Van Weemen and Schuurs (1972) and Engvall and Perlmann (1972). The underlying principle is the conjugation of antibodies or antigens to enzymes such that the immunological and enzymatic activities of each moiety are maintained. The degradation of a substrate by the enzyme, measured spectrophotometrically, is proportional to the concentration of the unknown "antibody" or "antigen" in the test solution. Apart from its convenience, ELISA has the following advantages; the labelled immunoreagents are stable for long periods, the precaution and disposal procedures required for the radioisotopes are unnecessary, the use of chromogenic substrates for the enzyme labels permits visual interpretation of test result. Competitive and non-competitive ELISA techniques are used for detection of either antigen or antibody. The competitive ELISA detects either the antigen (Belanger *et al.* 1973; Kato *et al.*, 1975) or the antibody (Hammarstrom, 1975). The non-competitive ELISA

technique is potentially more sensitive and widely used. However there are several variations such as direct method for detection of antibody (Engvall and Perlmann, 1972), double antibody sandwich method for detection of antigen and the antibody capture assay for detection of class specific immunoglobulin. The ELISA can be used as qualitative as well as quantitative assays for antibodies. In addition it provides a rapid method of diagnosing a wide variety of viral, fungal and protozoal infection with strain specificity where necessary. WHO (1974) reported that ELISA has been used widely for the detection and measurement of antibodies in response to erythrocytic stages of malaria infection and Egan *et al.* (1995) measured antibody responses to MSP1 using the microplate ELISA.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

This study was conducted at Dodowa, the capital of the Dangme West District of the Greater Accra region of Ghana. The district lies within longitudes 0° 5' and 0° 20' E and latitude 5° 40' and 6° 19' N. It is approximately 25 kilometres from Accra, the capital town of Ghana and is located between the coastal savannah and the secondary forest with few streams that flow most of the year. The forest is being depleted rapidly as a result of intense farming and other economic exploitation, hence the vegetation is now a mixture of few large trees with grass covering most of the area. Dodowa is gradually developing the characteristics of an urban community with greater accessibility to antimalarial drugs (Afari *et al* 1992).

Dodowa experiences two weather seasons, dry and wet. The rainy season runs from April/May to October/November and the dry season from November/December to March. The area has two peaks of rainfall during the year, the first occurs in June/July and the second in September. Rainfall is high in the forest areas and ranges between 77mm and 87mm per month. Humidity is highest, about 90%, in the rainy season and at its lowest, about 70%, during the dry season.

Malaria transmission occurs throughout the year, but is highest during or immediately after the major and minor rainy seasons, and lowest in the dry seasons. The transmission is considered as stable because it does not vary significantly from year to year. The incidence rate of clinical malaria is 106/1000 population per month and individuals are exposed to 22 infective bites per year with 98% of the infections caused by *P. falciparum* (Afari *et al.*, 1995).

3.2 Study population

Dodowa has a total population of 6,558 based on a 1992 census (Afari *et al* 1992). Children of ages of 5 years or below (≤5) and, those between 6 and 9 years (6-9) constitute 17% and 16.4% of the population respectively. The study population consisted of a cohort of 300 children within the ages of 3-15 years. They were followed as part of a longitudinal sero-epidemiological study during the period April 1994 to August 1995.

3.3 Ethical considerations

Informed consent for participation of the children was obtained from their parents after information had been given in the local language. The Ethical Committee of Ministry of Health, Ghana approved the study.

3.4 Study design

A target population in this longitudinal cohort study was selected after screening, using the metabisulphite test to exclude all those who had sickle cell trait or sickle cell disease. The children were followed with weekly clinical examination, monthly capillary bleeding (finger prick). Blood films (thick and thin) were prepared in the field from children with temperature ≥ 37.5°C and at monthly bleeding by an expert microscopist. The slides were transported to Noguchi Memorial Institute for Medical Research (NMIMR) and examined. The *Plasmodium* parasite density was determined after counting the number of parasites per 300 white blood cells (WBC) in the Giemsa stained thick films and multiplying by 8000/300 as an approximation of parasite count per microlitre. All negative slides were re-examined by counting up to 1000 WBC The children were classified into symptomatic and asymptomatic. An asymptomatic child had parasites in the blood in the absence of fever or other clinical signs of malaria such as rigors/chills vomiting, convulsion and musculo-skeletal pain. A symptomatic malaria patient had a positive blood slide together with temperature greater than 37.5°C with or without additional symptoms. Patients were excluded if they had signs of other diseases like throat and ear infections. Uninfected children had no detectable parasitaemia. For the present study only samples having *P. falciparum* for three consecutive months were included. The samples were grouped as follows: children who were asymptomatic for three consecutive months (A A A), those who were asymptomatic in the first and third month but symptomatic in the second month (A S A).

3.4.1 Sample collection

Each time a blood film was prepared for diagnosis of malaria, 50µl of blood from the individual was spotted onto filter paper (Whatman #5) after a finger prick and air dried. After drying, the samples were placed individually in small plastic bags, containing anhydrous silica gel and stored at -20°C until ready for use. Similarly blood samples had been collected from malaria patients (children) at University of Ghana, Legon Hospital for preliminary studies in standardising the PCR methods and also to determine its sensitivity. Venous blood was collected in heparin, centrifuged and blood plasma taken.

3.5 Reagents

The details of reagents used and their sources are provided as follows

3.5.1 Enzymes

Taq polymerase was supplied as a set with 10x buffer solution $MgCl_2$ (50 mM) and a 1% WT detergent (GIBCOBRL Life Technologies, USA).

3.5.2 Deoxyribonucleic triphosphate (dNTPs)

The four deoxyribonucleotides, deoxyadenosine triphosphate (dATP), deoxycytosine triphosphate (dCTP) deoxyguanidine triphosphate (dGTP) and deoxythymidine triphosphate (dTTP) were obtained from Boehringer Mannheim GmbH, Germany.

3.5.3 Oligonucleotide primers

Two sets of oligonucleotide primers designed to flank block 2 terminals of merozoite surface protein (MSP-1) gene (Table 1) were used. The sequences of the oligoprimers are the same as that published by Ranford-Cartwright *et al.* (1993) and was purchased from OSWEL DNA Services, U.K.

Primer	(bp) Length	Sequence (5' to 3')	Position
O_1	26	'CACATGAAAGTTATCAAGAACTTGTC'	5` MSP1 outer
O_2	22	'GTACGTCTAATTCATTTGCACG'	3` MSP1 outer
N_1	20	'GCAGTATTGACAGGTTATGG'	5` MSP1 inner
N_2	18	'GATTGAAAGGTATTTGAC'	3` MSP1 inner

Table 1 DNA sequences of oligonucleotide primers used for PCR amplification of alleles of MSP1 gene.

3.5.4 DNA molecular weight marker

The following DNA molecular weight markers were used as appropriate.

1200bp, 1300bp, 1400bp, 1500bp, and 2072bp

DNA molecular weight marker VI[2]

This marker was supplied by Boehringer Mannheim GmbH, Germany. It consisted of a mixture of pBR328, cleaved with *Bgl I*, pBR328 DNA and *Hinf I*. It generated 12 fragments of the following lengths.

154bp, 220bp, 234bp, 298bp 394bp, 453bp, 517bp, 653bp, 1033bp, 1230bp, 1766bp, and 2176bp

3.6 Standard solutions

Details of all solutions prepared can be found in Appendix 1.

3.7 Isolation of parasite DNA from blood spotted filter paper

The method used in the extraction of malarial parasite DNA from blood spot on filter papers was a modification of a protocol used by Walsh *et al.* (1991). Eppendorf tubes (1.5 ml) were labelled with the corresponding recognition codes. Two spots of blood stained filter paper covering a total area of 11.3mm^2 were excised by a precision hole puncher and put into Eppendorf tubes containing a total volume of 200µl of 10% Chelex (w/v) solution which was continuously stirred while being pipetted. The contents were thoroughly mixed and incubated at 56°C for 30 minutes in a water bath whilst shaking. They were then vortexed for 5 seconds and incubated in boiling water for 10 minutes. They were vortexed for a further 10 seconds and spun in a microcentrifuge (Kubota, Japan) at 15,000 r.p.m. for 10 minutes. The supernatant was collected and the filter paper discarded. The

stirred while being pipetted. The contents were thoroughly mixed and incubated at 56°C for 30 minutes in a water bath whilst shaking. They were then vortexed for 5 seconds and incubated in boiling water for 10 minutes. They were vortexed for a further 10 seconds and spun in a microcentrifuge (Kubota, Japan) at 15,000 r p m. for 10 minutes. The supernatant was collected and the filter paper discarded. The supernatant obtained was centrifuged 2x and then transferred into 0.5 ml Eppendorf tubes for storage at -45°C until ready to use. Cross-contamination of samples was avoided by treating the hole puncher and forceps used with 5M hydrochloric acid followed by 5M sodium hydroxide after each sample to prevent carry-over of DNA from one sample to another.

3.8 PCR amplification

A typical reaction mix for a 100µl reaction contained the following: 1x PCR buffer (20mM Tris-HCl pH 8.4, 50mM KCl), 200µM each of Deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, dTTP), 1.5mM of Magnesium chloride, 0.8µM of each primer, 0.5% (v/v) of detergent (W-1), 2.5 unit *Taq* DNA polymerase and 5µl of extracted DNA solution. For the nested PCR only 1-2µl of the first round PCR product was used as DNA template. For the present study a reaction volume of 50µl was used and amplifications carried out in 0.5µl Eppendorf tubes. The contents were thoroughly mixed, overlaid with mineral oil (Sigma Chemical Corporation) and spun for 5 seconds. Mineral oil was added to prevent evaporation and refluxing of the reaction mix during thermocycling.

The PCR assays were carried out using a thermal cycler (Techne Progene, USA) under the following conditions; an initial cycle of denaturation at 94°C for 2 minutes, primer annealing at 50°C for 25 sec and extension at 68°C for 2 minutes 30 sec. followed by 29 cycles of denaturation at 94°C for 25 sec, annealing at 50°C for 30 sec and extension at 68°C for 2 minutes 30 sec. The reaction was concluded with a cycle of an extension time of 10 minutes and the reaction product was stored at 4°C until use. The same cycling conditions were used for the nested PCR.

3.9 Agarose gel electrophoresis

The agarose gel was prepared as described by (Sambrook *et al.*, 1989). Briefly, 1.5 g of agarose granules was weighed into an Erlenmeyer flask to which 100ml of 1 x Tris acetate EDTA buffer (TAE) was added. It was covered with a piece of perforated cling film and heated in a microwave oven for 2 minutes. Ethidium-bromide (5µl) was added to the mixture and then cooled gently. A gel tray was prepared by placing the comb to form the wells and sticking strips of tape to the open sides of the tray. The agarose solution was poured into the tray and left for about an hour to solidify. The comb and tape were removed and the tray put in a horizontal Minigel electrophoretic system, (Minigel submarine, BioRad) containing the same buffer as the gel. The loading buffer (2 0µl 5x Orange G) was mixed with 10µl of the PCR product on a piece of parafilm before loading. About 3-5µl of the molecular weight markers was used. Electrophoresis was carried out at 80V for two hours and the gel viewed on an UV transilluminator (T.M-20, USA) and photographed using a Polaroid camera fitted with an orange filter.

agarose gel using graphical method (Appendix 2) The fragment sizes of the markers used during the study are listed in section 3.5.4. Where DNA fragments were very close on agarose gel, polyacrylamide gel was used to further resolved them but it was not used to estimate DNA fragment sizes

3.10 Discontinuous non-denaturing polyacrylamide gel electrophoresis

This method was used to obtain maximum separation of bands. Electrophoresis was performed using a vertical Mini-Protean Dual Slab Cell System (ATTO, Japan). The glass plate was sandwiched and the equipment was assembled and operated according to the protocols supplied by the manufacturer.

The separating gel was prepared by mixing 20 ml of 4.5% gel solution and 1500µl of 1.5% Ammonium persulphate (APS) (Appendix 1), degassed for 15 minutes in a vacuum chamber (Nalgene, Sybron corporation) before 15µl of N N N N - Tetramethylethylenediamine (TEMED) was added. Similarly, the stacking gel was prepared by adding 500µl of 1.5% APS to 5% stacking gel solution (Appendix 1), degassed for 15 minutes before 4µl TEMED was finally added. The separating gel solution was poured between the two plates to the desired level and immediately overlaid with water

The gel was set usually within 40 minutes and the water was drained off. The stacking gel solution was poured onto the separating gel and the comb inserted immediately. It was pre-run at 150V and 30 mA for 30 minutes in 1 x Tris borate EDTA (TBE) buffer, after the wells had been thoroughly cleaned by washing with

solution was poured between the two plates to the desired level and immediately overlaid with water.

The gel was set usually within 40 minutes and the water was drained off. The stacking gel solution was poured onto the separating gel and the comb inserted immediately. It was pre-run at 150V and 30 mA for 30 minutes in 1 x Tris borate EDTA (TBE) buffer, after the wells had been thoroughly cleaned by washing with distilled water to remove unpolymerized acrylamide. The wells were loaded with a mixture of 5µl of each of the PCR products and orange G x (1). The two outer terminal wells were loaded with 4µl of the desired molecular weight marker. The electrophoresis was run for 1.5 hours at 120V and 15mA. Thereafter, the apparatus was dismantled and the gel removed for silver staining.

3.10.1 Detection of DNA products using silver staining method

The polyacrylamide gel was fixed in a solution of 10% ethanol and 0.5% glacial acetic acid for 25 minutes. It was transferred to a solution of 11mM $AgNO_3$ for 25 minutes and then washed twice with tap water. It was then developed in 15 ml of 5M NaOH and 0.8% Formalin solution. When the DNA bands were visible, placing the gel in 10% glacial acetic acid stopped the reaction. Finally the stop solution was poured off and the gel washed with tap water and placed on a light box and photographed using a Polaroid Camera.

3.11 Sensitivity of PCR assay

The aim of this experiment was to determine the lowest level of parasitaemia that

the PCR could detect under our laboratory conditions. This was done before analysis of the samples from Dodowa. A small drop from blood which was collected in 10 ml containers from patients who reported at the University of Ghana hospital, Legon, was placed on a glass slide (25 mm x 75 mm) and another slide was used to spread the blood evenly to produce a thin smear which was air dried. The thin film preparation was fixed by immersing the slide in absolute methyl alcohol for 30 seconds and the 5% Giemsa stain solution was then poured on the slide whilst it was placed flat on two glass rods and left for 15 minutes. The thick film was also prepared by placing three small drops of blood in the middle of a slide and the drops were spread to form a film of blood. It was allowed to dry at room temperature and stained with 5% Giemsa. The stain on both slides was gently washed off with tap water and the slide was left upright to dry. The thick film was however not fixed. Both the thick and thin blood films were examined using an Olympus CH-2, microscope. Infected as well as uninfected red blood cells (RBC) were counted in eleven oil immersion fields of standardised thin blood film at a magnification of 1000x and the parasitaemia was calculated as ratio of the infected to uninfected RBC counted. The total number of RBC was estimated using a modified protocol described by Lynch *et al* (1969). The results obtained were later checked with that using an automated haematological analyzer counter (MicroDiff 18, Coulter, USA).

For manual estimation, Sahli's pipette was used to take 0.02 ml blood and washed into 4 ml of diluting solution (40% formaldehyde and 3% (w/v) trisodium citrate) to give a 1 in 201 dilution. The diluted blood was then mixed thoroughly using a

whirl mixer (Denley Greiner, England) and the counting chamber was filled using a capillary tube. Eighty squares of the counting chamber were examined by microscopy. The total number of RBC was estimated using the formula R x 200 x1/0.02, where R is the dilution factor. The percentage parasitaemia was then calculated.

Once the parasitaemia has been determined the infected blood was then serially diluted in ten steps with blood of non-immune uninfected Danish nationals. Fifty microlitre blood of each of infected and unifected blood as well as the serially diluted was spotted on filter papers (Whitman #5), air dried and stored at -45°C

DNA of the parasite was then extracted from each filter paper and PCR carried out using the methods and the reaction conditions described (sections 3.7 and 3.8). For all reactions both positive and negative controls were carried out and the results of the PCR were analysed as previously described (section 3.9).

3.12 Enzyme-linked immunosorbent assay (ELISA)

The ELISA assay performed to measure the anti-MSP1 antibodies against *P falciparum* antigens ($MSP1_{19}$) was a modification of the protocol described by Riley *et al.* (1991). The modified method is routinely used in the Immunology Unit of the Noguchi Memorial Institute for Medical Research, and for the present study was targeted at the $PfMSP1_{19}$. Dynatech Microtitre plates were coated with 1µg/ml of the antigen in carbonate buffer (pH-9.6) (constructed with glutathione) donated by Dr Eleanor Riley, of the University of Edinburgh. Wells A-1 and A-2,

which were used as blanks, were not coated. The plates were incubated at 4°C overnight, washed three times with phosphate buffered saline (PBS)/Tween (pH-7.4) using an autowasher (EL 404 BIOTEK, USA), and then blocked with 5% bovine serum albumin (BSA) in tween-20. The blocking was performed at 37°C for 2 hours followed by three times washing with PBS tween. Plasma samples were diluted 1:100 in BSA, added in duplicates and then incubated at 37°C for 1 hour. After washing three more times, a 1:2000 dilution of anti-human IgG alkaline phosphatase conjugate (Sigma, USA) was added and the plates incubated at 37°C for 1 hour. The plates were washed three times and then developed with 1μg/ml p-Nitrophenyl Phosphate (PNP). The reaction was stopped with 3M NaOH after incubating for 25 minutes at room temperature in the dark. The absorbance was read at 405 nm using microplate reader MTP-32 (Corona Electric, Japan). The minimum significant value was established from the mean of the negative controls (x) + 2 standard deviation. The cut-off point of optical density (OD)< 0.3 indicating negative and OD>0.4 as positive were used in the analysis that sought to determine whether there was any association between increased antibodies and clinical protection from malaria. In this study, negative control sera were obtained from non-immune and uninfected Danish nationals who have never been exposed to malaria. The positive control sera were obtained from patients who have had several malaria attacks.

CHAPTER FOUR

RESULTS

4.1 Sensitivity of PCR for *P. falciparum* detection

The aim of this experiment was to determine the least detectable number of *Plasmodium falciparum* from filter paper blood using PCR assay based on the amplification of the MSP1 gene. As shown in Figure 4, the bands represent a DNA fragment size of 600 bp. Initially the blood contained 218250 parasites per microlitre

but was diluted. The same DNA fragment size was expressed but the band gradually became faint with increasing dilution. Consequently, beyond 1: 100,000 dilution of the test blood no band was observed in the gel (Figure 4).

Based on the dilution the sensitivity of the PCR technique employed in this study was calculated to be 22 parasites/µl of blood using the outer primers for amplification and agarose gel for analysis but with polyacrylamide gel analysis as low as 2.2 parasites/µl of blood could be revealed.

4.2 Comparison of PCR and microscopy in detection of *P. falcipasrum*

The PCR assay was assessed using microscopically positive and negative samples as shown in Table 2. A total of 197 samples obtained from a subpopulation of 38 children were analysed to establish the sensitivity of the PCR assay using outer and inner primers (Table 3)

Microscopic examination of 197 giemsa-stained blood films showed that 194 contained *Plasmodium falciparum* and 3 had no parasites. The PCR assay confirmed 160 microscopically positive samples when outer primers were used and missed 34 samples. Similarly outer and nested PCR confirmed 1 out of 3 microscopically negative samples while 2 were positive (Table 3). This gave a relative specificity of 33.3%. In all, the outer primer showed 161 samples positive and 36 samples negative. The nested PCR performed on 34 microspically positive but nagative when outer primers were used for amplification indicated that 32 of the samples were really nagative and two were positive. In summary therefore, a total of 192 out of 194 microscopically positive samples were PCR positive representing 99% sensitivity.

4.3 Allelic type(s) in individuals with consecutive asymptomatic (A_1) symptomatic (S) and asymptomatic (A_2) cases of malaria status

The purpose of this work was to find out if symptomatic cases of malaria are limited to certain MSP1 allelic types. To determine this, blood on filter paper of individuals who exhibited consecutive asymptomatic, symptomatic and asymptomatic malaria in the dry and wet seasons were used. Table 4A and 4B summarise the results obtained for the two seasons and showed the sizes of the amplified MSP1 DNA fragment sizes and their corresponding parasite densities during wet and dry seasons. Different MSP1 types (Figures 5 and 7) were associated with malaria episodes.

In general there was a switch of allelic type (determined by size differences) when

an asymptomatic child suddenly became symptomatic. In all, 8 children showed this A_1SA_2 phenomenon of which 6 (75%) had allelic type switch over when they experienced malaria episode. The list showing the switch over of the corresponding children is provided in Tables 4A and 4B; namely 1(ABC) – 546bp to 596bp, 13(ABC) – 615bp to 602bp, 20(EFG) – 615bp to 596bp, 219(CDE) – 423bp to 376bp, 276(DEF) – 447bp to 531bp and 124(ABC)- 596bp to 631bp. However 2/8 (25%) children did not show the switch over when they experienced a clinical malaria episode but maintained the same allelic types. The DNA fragment sizes of the samples belonging to these children were 284(EFG) – 477bp and 139(ABC) – 582bp.

It was observed that the allelic type(s) corresponding to 596bp was associated with malaria attacks in two children 1(ABC) and 20(EFG). However an allelic type of the same size was also found in an asymptomatic child 124(ABC). Children 13(ABC) and 20(EFG) had allelic type of 615bp in the asymptomatic blood samples that preceded the malaria episode but their corresponding symptomatic blood showed different DNA fragment sizes of 602bp and 596bp, respectively. In the child identified as 13(ABC), the same DNA fragment size of 602bp that caused clinical attacks remained even after recovery. Whereas allelic type of DNA fragment size of 477bp was found in asymptomatic blood sample of child 276(DEF), the ensuing symptomatic blood sample showed different allele of DNA fragment size of 531bp. Similarly the allele of DNA fragment sizes of 477bp in the asymptomatic blood of 284(EFG) caused clinical malaria the following month. The same DNA fragment size remained a month after recovery. The MSP1 allele

of 596bp was associated with clinical malaria during the wet season but was associated with asymptomatic malaria in the following dry season in sample 124(ABC). The DNA fragment size of 582bp in the asymptomatic blood sample of 139(ABC) remained unchanged the following month. However it changed to 613bp the next month he recovered. There were instances where reversion to allelic type of the preceding asymptomatic phase showed up after malaria attack. Figure 6 illustrates this clearly in children identified as 1 and 13. In other instances, such as in child 13, the allelic type found in attack phase was the same as that found when the child was asymptomatic (Figure 8).

There were no specific MSP1 allelic types associated with a particular season. Nontheless there were diverse forms of the MSP1 gene (Figure7)

Generally there was an increase in parasite densities when previously asymptomatic children experienced clinical malaria except 124(ABC) who showed a slight decrease in parasite density. The results (see Table 4A and 4B) indicates a significant increase ($p = 0.017$, Mann Whitney rank test and $p< 0.05$, Student Newman Keuls method). There was however no association {χ^2 (Yates corrected), $p = 0.134$} of clinical malaria with acquisition of new parasite strain.

4.4 MSP1 allelic type(s) in individuals with three consecutive asymptomatic malaria status

The details of the results were shown in Table 5A and 5B. In all 23 children were examined of which 6 showed no change at all in allelic types. Switch over involving two allelic types were observed in sample from child 13 and in samples

obtained from other three children, all the alleles were different. Multiple infections of two alleles in blood samples were observed in five instances (Table 5A and 5B)

4.5 Parasitaemia levels during symptomatic and asymptomatic malaria cases

Generally there was an increase in parasite densities when previously asymptomatic children experienced clinical malaria (Table 4A and 4B). This was observed in the samples belonging to the following 1(ABC) 400-17469, 13(ABC) 2773-4507, 20(EFG) 160-1093, 284(EFG) 773-7573,139(ABC) 240-933. However there was only one sample 124(ABC) which showed slight decrease in parasite density. There was a significant increase ($p=0.17$, Mann Whitney rank test and $p< 0.05$ Student Newman Keuls method) In parasitaemia during symptomatic malaria status of the individuals. In three consecutive asymptomatic cases, there was generally no increase in parasite densities during the three months period (see Tables 5A and 5B). There was no significant increase ($p=0.105$ Friedman repeated ANOVA on ranks) in parasites densities between any of the months.

4.6 Anti-MSP1 Immunoglobulin G (IgG) responses and protection from malaria attack

The purpose of this experiment was to find out whether anti-MSP1 antibodies status of individuals are altered during malaria attacks and correlates with clinical protection. Anti-MSP1 antibody responses to the 19KDa C-terminal part of the MSP1 in school children before and after malaria attack showed that 14 out of the total 27 children were positive (Table 6). Nine of the children fell within the ages

of 3 to 4 years whereas eighteen were ≥ 5 years. Four children out of the 9 children aged 3-4 years had no anti -MSP1 antibodies before malaria attack but three of them produced anti-MSP1 antibodies after malaria attack indicating sero-conversion of 75% for this age group. The five children who were positive for anti-MSP1 antibodies before attack had no change in their anti MSP1 IgG titres. Nine out of the eighteen children ≥ 5 years were positive and the other 9 negative for anti-MSP1 antibodies before malaria attack. However after malaria attack two children become negative for IgG (OD_{450nm} = 0.984 to 0.300, and 0.489 to 0.326) constituting 22%. In all 75% negative to positive sero-conversion and 22% positive to negative sero-conversion were shown.

TABLES

Table 2. Determination of percentage parasitaemia by microscopy (100X magnification) in 11 fields of a single blood film

A. Determination of parasitaemia by microscopy

Field	Total number of red cells	Infected red cells
1	116	5
2	121	4
3	100	7
4	68	4
5	71	2
6	106	6
7	68	4
8	67	4
9	100	6
10	109	4
11	125	5
Total	1051	51

B. Determination of PCR sensitivity

Using the formula,

$$\text{Percentage parasiteamia} = \frac{\text{No. of infected RBC X 100}}{\text{Total no. of RBC}}$$

$$= \frac{51 \text{ X } 100\%}{1051}$$

$$= 4.85\%$$

Parasite density = % parasitaemia x Total RBC count

$4.85\% \times 4.50 \times 10^{6}/\mu l$

$= 218250 /\mu l$

	Positive						Negative	
Dilution	1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Parasite density	21850	21825	2183	218	22	2.2	0.22	0.022

The PCR method can detect parasites in blood samples with 2.2 parasites per microlitre.

Table 3: Relative sensitivity of PCR to Microscopy for detection of *P. falciparum* in blood samples.

Microscopic Examination	Single PCR (O_1+O_2)		Nested PCR (N_1+N_2)	
	Negative	Positive	Negative	Positive
Positive (n=194)	34	160	2	32
Negative (n=3)	2	1	1	1
Total (n=197)	36	161	3	33

O_1 and O_2 = Outer primers

$N_1$and N_2 = inner primers

Table 4A and 4B: Comparison of MSP1fragment sizes from three consecutive asymptomatic, symptomatic and asymptomatic samples and their corresponding parasite densities during dry and wet seasons.

4A- wet season

Study number	Molecular Weight (bp) of MSP1 allelic types			Parasite density. (No. /µl blood)			New allelic type
	A_1	S	A_2	A_1	S	A_2	
1 (ABC)	546	596	546	400	17469*	133	Yes
13 (ABC)	615	602	602	2773	4507*	320	Yes
20 (EFG)	615	596	589	160	37707*	1040	Yes
219 (CDE)•	422	376	398	27	880*	133	Yes
276 (DEF)•	447	531	516	27	1093*	160	Yes
284 (EFG)•	477	477	477	773	7573*	453	No

4B- Dry season

Study number	Molecular Weight (bp)			Parasite density.			New allelic type
	A_1	S	A_2	A_1	S	A_2	
124 (ABC)	596	631	668	1707	1147*	27	Yes
139 (ABC)	582	582	613	240	933*	1147	Yes

• = Nested PCR using inner primers, A_1 = Asymptomatic cases before malaria episode, S = Symptomatic cases, A_2 = Asymptomatic cases after malaria episode.

* = There was statistically significant difference ($p = 0.017$) using Mann Whitney Rank sum test and also All Pairwise Multiple Comparison (Student Newman Keuls Method, $p < 0.05$) for the parasite densities.

There was no association of clinical malaria with acquisition (χ^2 Yates corrected $p = 0.134$).

The alphabets such as A, B, C etc correspond to the months during which blood samples were taken. The first month was taken as A.

Table 5

Comparison of molecular sizes of MSP1 allelic types for blood samples taken in three consecutive months from asymptomatic individuals and their corresponding parasite densities.

5A Wet season

Study number	Molecular Weight (bp)			Parasite density.			New allelic type
	A_1	A_2	A_3	A_1*	A_2*	A_3*	
20 (ABC)	614	602	602	480	12400	269	Yes/No
106 (DEF)	562	562	516	560	80	267	No/Yes
158 (ABC)	562	562	546	213	2000	33947	No /Yes
159 (ABC)	624	638	638	8347	2267	9200	Yes /No
359 (DFE)	579	579	579	320	640	2987	No
64 (DFE)	562	562	562	160	53	13	No
114 (DFE)	631	631	668	3707	80	9200	No Yes
345 (CDE)	668	668	749/631	267	1040	2720	No Yes
351 (ABC)	596	596/562	596	15093	6400	17333	(No)(Yes)

A_1 =Asymptomatic cases before malaria
S =Symptomatic malaria cases
A_2 =Asymptomatic cases after malaria

5B- Dry season:

Study number	Molecular Weight (bp)			Parasite density.			New allelic type
	A_1	A_2	A_3	A_1*	A_2*	A_3*	
72 (ABC)	562	562	649	720	80	3067	No /Yes
192 (ABC)	596	596	596	373	667	453	No
240 (DEF)	596	596	596	240	53	1040	No
266 (DFE)	596	569	596	540	80	160	Yes
284(ABC)	559	649/559	631/559	4667	560	2080	(Yes)(No)
285(ABC)	560	617/519	560	640	640	53	Yes
360(ABC)	638	638	575	320	9760	560	Yes/No
421(ABC)	646	646	646	9040	80	1547	No
422(ABC)	596	596	582	1173	1973	347	No/Yes
85 (CDE)	546	579	546	80	1040	187	Yes
92 (ABC)	579	579	546	80	267	933	No/Yes
214(ABC)	550	550	582	1867	902	373	No/Yes
291(ABC)	649	649	649	213	427	267	No/Yes
354(ABC)	562	562	516	1040	2560	160	No/Yes

* = There was no statistically significant difference ($p = 0.105$) in parasite densities using Friedman repeated ANOVA on ranks.

Table 6: Anti-MSP-1 antibodies in different age groups of school children before and after malaria attack.

Age Years	Number Tested	Antibody Status		Sero-Conversion(%)	
		Positive	Negative	-/+	+/-
3-4	9	5	4(3)[b]	3/4(75%)	0
≥5	18	9(2)[c]	9	0	2/9(22%)
Total	27	14(2)[c]	13(3)[b]	3/13	2/14

a = Presence or absence of anti MSP1 antibodies before and after malaria attack.

b = Number of individual with anti MSP 1 antibodies after malaria attack.

-/+ = Production of anti MSP1 antibodies in previously negative individuals.

+/- = Loss of anti MSP1 antibodies in previously positive individuals.

c = Number of individuals without anti-MSP1 antibodies after malaria attack.

Optical density (OD_{450nm}) = (OD < 0.3 – negative, OD > 0.4 – positive)

There was no statistically significant difference (p = 0.182) using McNemar's test

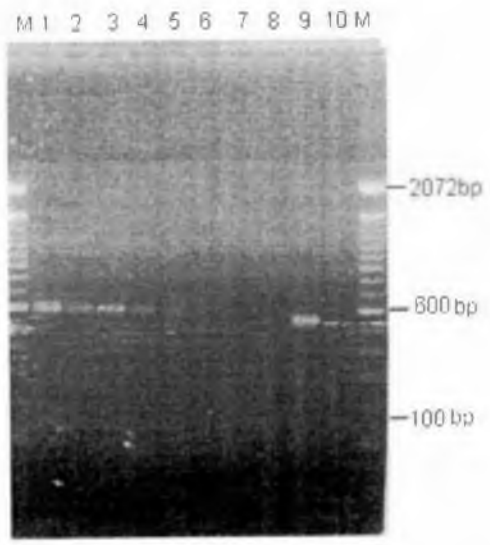


Figure 4: Sensitivity of PCR for detection of blood stage malaria parasites. Amplifications were performed on serially diluted samples of known parasitaemia using outer primers. Lanes M show makers. Lanes 2- 8 represent ten fold serial dilution. Lanes 9 and 10 are positive and negative controls respectively.

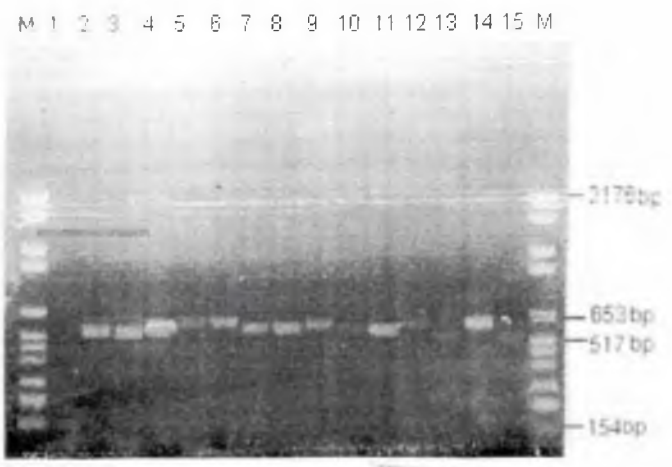


Figure 5: An illustration of the diversity of MSP allelic types found at Dodowa. Samples were obtained from three patients in both wet and dry seasons. Lanes 3-9 show patient number 20. 10-12 represent patients number 13 and 13-15 patient number 1. Lanes 1and 2 are negative and positive controls respectively. Lanes 5,6 and 15 are dry season samples whereas 3,4,7,8,9,10,11,12,13 and 14 are wet season samples. Lanes 11 and 14 from patient showed clinical malaria.

Figure 6: The distribution of MSP1 variants in two patients before and after malaria attacks. Lanes 1,2 and 3 are MSP1 alleles of patient 13 and 4,5,6 are those of patient 1

A = Asymptomatic. S = Symptomatic, M = Marker

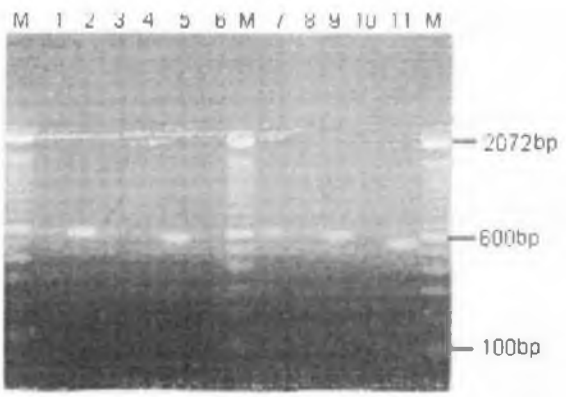


Figure 7: Ethidium bromide stained 1.5% agarose gel of PCR product of *P falciparum* showing MSP1 allelic types of symptomatic cases n = 7. Lanes M show marker, 1,2,3 and 7 are dry season samples. Lanes 4,8 and 10 are asymptomatic cases. The gel shows different allelic types of MSP1 gene that were associated with malaria attacks.

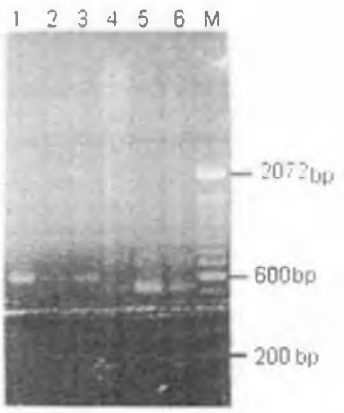


Figure 8: An illustration of MSP1 allelic type in an asymptomatic subject during three consecutive sampling. The first three lanes represent amplification using outer primers and ensuing three lanes show amplification using inner primers.

CHAPTER FIVE

DISCUSSION

5.1 Methodology

It has been shown that the PCR method was used with success to detect *P falciparum* in blood spots on filter paper using a rapid and simple method of boiling to isolate parasite DNA. The results obtained even showed that the first round of PCR using only the outer primers for MSP1 alleles detection was sensitive enough to analyse most of the samples obtained by simple blood spot on filter paper.

As shown in Figure 2 as low as 22 detect *P. falciparum* parasites/µl were detectable in agarose gel and 2.2 parasites /µl in polyacrylamide gel confirming the superiority of polyacrylamide gels over agarose gels (Dawson *et al.*, 1996). In summary, the observed limits of detection were between 0.1% - 0.01% parasitaemia using the outer primers. However, the minimum detectable number of *P. falciparum* using outer primers was 25/µl obtained by others (Ntoumi *et al.* 1995). The nested PCR detected even lower levels of parasitaemia than the first round PCR in conformity with Foley *et al.*(1992) who showed through a nested PCR the scaling down of the minimum detection level from 500 parasites/µl to 200 parasites/µl using the MSP1 as primer set. Using microscopy as the reference standard the PCR gave a relative sensitivity of 99% and a relative 33.3% specificity when parasitaemia was as 2.2 -

22 parasites per microlitre of blood. The nested PCR missed only two (2) microscopy - positive specimen (with 650 and1400 parasites/μl). The reason for this is unclear as other samples with relatively lower levels of parasitaemia were successfully amplified. There are several possible explanations as to why the PCR was not successful in the first instance. First, there could have been possible degradation of genomic DNA. Secondly, the concentration of the isolated parasite DNA might have been scanty below the detection limit of PCR. The negative sample could have resulted from a mutation in the sequence targeted by the primers but was not investigated. It could also be attributed to very low levels of parasitaemiae which was overestimated. There was one sample which was both negative by microscopy and PCR indicating the absence of parasitaemiae in the blood sample. Two microscopy negative samples were found positive and this confirmed the superiority of PCR in detecting parasitaemia (Snounou *et al.*, 1993). To summarise, 99% relative sensitivity and 33.3% specificity were obtained when PCR was performed using isolated parasite DNA from dried blood on filter paper. These levels were adequate for the present study. The use of dried blood spot was an improvement over other studies that achieved similar results but used whole blood that needed thawing.

5.2 Allelic forms

PCR typing method was used to distinguish the different MSP-1 allelic types in the locality as shown in figures 5-7. It was observed that there were 35 MSP-1 allelic types similar to the 34 distinct MSP-1 alleles that were found in Senegalese children (Contamin *et al.*, 1996). In addition and the most common allele of DNA

size (596bp) did not exceed 15% of the total allelic types. This reflects the local transmission intensities of circulating *P. falciparum* strains.

It is possible that two parasites of the same allelic size polymorphism would differ from each other when more than one locus is examined. It is also possible that certain alleles, which were identical in size, could have varied DNA sequences. Conversely, the allelic types observed could be invalidated by placing the same allelic type into different size classes, stemmed from the estimation of the DNA fragment sizes of PCR products in agarose gel with an accuracy range within 10-15bp (Ranford-Cartwright *et al*, 1993). The absolute sizes of the PCR products were not critical to the interpretation of the main study. Comparison between three consecutive blood samples (either asymptomatic, symptomatic and asymptomatic or three consecutive asymptomatic cases) were carried out by examining the PCR products run in adjacent tracks of a single gel. Despite the limitation in estimating the DNA fragment sizes from a gel, the PCR products exhibited a wider range of sizes from 513bp to 749bp when outer primers were used, the nested PCR ranged from 376bp to 531bp. It was observed that 12.9% of the samples showed two multiple fragments size (Tables 5A and 5B). These were all asymptomatic samples, a situation consistent with the switching over model where infection with a new strain leads to clinical episode (Jeffery, 1996). The multiple allelic infections in individuals (Results 4.3) are probably of high transmission intensity in an area has been postulated by (Daubersies *et al.*, 1996) and Paul *et al.* (1998). It might also be due to the chronic nature of asymptomatic infections.

5.3 Seasonal variation

As shown in Figure 5 and 7 there was seasonal variation of allelic types. There were many allelic types during both dry and wet seasons. This confirms that there is substantial changes in composition of the parasite population in peripheral circulation irrespective of the season (Daubersies *et al.*, 1996, Paul *et al.*, 1998). Interestingly, the estimated DNA fragment sizes of the MSP-1 gene indicated no single allelic type contrasting with *Toxoplasma gondii* where the genetic make up of virulent parasite is remarkably homogenous (Sibley and Bothroyd, 1992).

5.4 Association of clinical malaria with new allele

The allelic types harboured by the children who initially were asymptomatic but suddenly became symptomatic in the subsequent month and then turned asymptomatic again after treatment revealed a change of allelic types compared with the initial asymptomatic sample. However there is no significant association (χ^2, p= 0.134) of acquisition of new allelic type with clinical attack. This contradicts the assertion that the onset of symptoms in chronically infected, previously asymptomatic individual, may be due to the introduction of new parasite (Contamin *et al.*, 1996). The clinical attack might have culminated from the random feeding habits of the female Anopheles mosquito and lack specific virulence strains. It is also influenced by different parasite interaction in the individual which change the course of infection (Riche, 1988, Snounou *et al*, 1992). It could be speculated that clinical malaria is mainly influenced by the cross-reaction of immunodominant epitopes and other repeated epitopes either within the same molecule or other parasite proteins (Theander, 1991). The cross-

reacting epitopes could interfere with the absorption of antibodies that are not essential for the survival of the parasite but divert immunological response from more important epitopes. This therefore promotes random advantage to any of the infecting allele.

Figure 4A for example supports the allelic changes that occur during malaria. Sample 1(CBA) showed reversion to the original asymptomatic allelic type a month after the treatment was observed. The possible but alternate interpretations other than renewed inoculation, is that the allelic type originally present was either relatively resistant to the anti malarial drug or it was in competition with allelic types which were dominant during malaria attack and hence escaped PCR detection. Similarly, in 13(CBA) the DNA fragment size of the original asymptomatic sample was different from the symptomatic sample. However one month after the treatment the DNA fragment size found in the blood sample had the same molecular size as the symptomatic sample. In addition, recrudescence as a result of drug resistance or inoculation of the same allelic type granting that equal molecular weight sizes correspond to genetic homogeneity could also be the cause.

5.5 Synchronization of high parasitaemia with malaria

The striking revelation from this study was the association of malaria episode with high parasitaemia (see Tables 4A, 4B, 5A and 5B). The observation is compatible

with the interpretation that clinical protection reduces with increased parasite density. This could stem from differences in the growth rate of various allelic types, resulting from the poor fitness of some types in certain children, such as poor invasion efficiency, impaired intraerythrocytic maturation, or slow replication rate and intense specific immune pressure, which would restrict parasite multiplication of certain allelic types while leaving other allelic types unaffected (Contamin *et al.*, 1996). Increased parasitaemia is significantly associated with clinical malaria (p= 0.017). This implies that the activities of non-specific and specific immune responses lag behind the unrestrained multiplication of the parasite.

No significant increase in parasite densities of three consecutive asymptomatic infections (p= 0.105) supports that symptomatic cases are synchronised with high parasitaemia. However other factor such as age, cross-reacting epitopes, immunosupression, previous exposure can influence the course of infection and the potential to cause clinical attacks (Theander, 1991; Ntoumi *et al.*, 1995).

5.6 Anti-MSP1 antibodies responses

Elucidation of the respective contribution of species-specific and nonspecific responses to control parasite propagation is a key element in our understanding of protection against malaria. To date, this has been difficult to study because the immune effectors contributing to parasite clearance in humans are unclear, and as a consequence, the MSP-1 target antigen supposedly offering clinical protection (Riley *et al.*, 1991; 1993) was investigated to unravel the biological role merozoite surface protein-1_{19} plays in the development of clinical protection from malaria

attack.

Antibody production during malaria parasite infection is age dependent in endemic population (Ntoumi *et al.*, 1995). Children develop antibodies against variant epitopes whereas adults develop antibodies against less but conserved epitopes (reviewed Theander, 1991). This explains why three children in the age group of 3-4 years showed 75% negative to positive sero-conversion in contrast to 22% positive to negative sero-conversion showed by those in the age group ≥ 5 years after malaria attack. It confirms Theander (1991) assertion that the rate of IgG synthesis is three times as high in unprotected individuals than protected one, in endemic areas and also seven times higher in non-immune people.

It is believed that those in the age group of ≥ 5 years have relatively matured non-specific immune systems. This accounts for the reduced anti-MSP1 production after malaria attack because the parasitized cells can be eliminated mainly by various leucocytic responses (Sheagren, *et al.*, 1970). Another possible explanation to the lost anti-MSP1 antibody production after malaria attack in two children in the age group ≥ 5years could be attributed to low immunogenicity of epitopes within C-terminal region of MSP1 or lack of adequate T-cell help for antibody production (Andrea *et al.*, 1997; George *et al.*, 1996; Venkatachalam *et al.*, 1995). Also the tendency to respond or not to respond to the C-terminal part of the MSP1 could depend on the host factors and the parasite ability to suppress the host immune response. Some MSP1 antibodies serve as smoke screen preventing the body from recognising the particular part of the molecule involved in

protection (Bouharoun-Tayoun and Druilhe, 1992). It may be possible that cross-reaction between different parasites or within the same parasite molecule simultaneously infecting the same individual (Riche, 1988; Snounou *et al.*, 1992, Anders, 1986) resulted in rapid decline of the immune responses to produce antibodies (Staalsoe and Hviid, 1998). It could be speculated that those who lost antibody when they experienced malaria attack might have had the episode caused by mixed infection. In this case there were cross-reactions that interfered with the maturation of the high affinity antibodies directed against $MSP1_{19}$ and with the absorption of antibodies on the antigen, thus simply diverting immunological responses from more important epitopes (Theander, 1991). In summary, there was no significant correlation ($p= 0.182$) of anti-MSP1 antibodies production with adequate protective immunity.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

In conclusion, the PCR-based assay showed a high relative sensitivity of 99% with 33.3% specificity. The least detectable parasites per microliter of blood was 2.2 using outer primer set. None of the 35 MSP1 allelic types could be said to cause clinical malaria at all times. Though clinical malaria was associated with significantly high parasitaemia, there was no correlation of acquisition of new MSP1 allelic type with clinical malaria ($p= 0.134$). Similarly, anti-MSP1 IgG antibody production was not associated with protection from malaria ($p= 0.182$)

6.2 Recommendations

It is therefore recommended that:

1. Change in allelic type alone cannot does not appear to account for clinical malaria therefore any investigation related to strain specific malaria should take into account all other factors.
2. allelic type specific antigens should be developed to assay antibodies in order to determine the relationship between strain specific responses and clinical malaria.

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Appendix 1

Standard Solution.

The following standard solution were prepared using sterile double distilled water (sddw). Where appropriate, the solutions were autoclaved at 121lbs for 15 minutes in Eyela Autoclave(Rikikkaki, Tokyo).

DNA Extraction.

CHELEX (20%)

20g of Chelex dissolved in some amount of sddw and made up to 100ml.

5M HCl

7.68ml of concentrated HCl was measured and diluted to 100ml with sddw.

5M NaOH

4g of NaOH weighed and dissolved in sddw to make 100ml

Solutions for Electrophoresis.

Agarose gels.

10x TAE buffer

242g Tris base, 57.1ml glacial acetic acid, 100ml 0.5M EDTA, pH adjusted to 7.7 (with glacial acetic acid) and the volume made to 1000ml ddw

10x TBE 100g/l Tris base 55g/l Boric acid acid 9.3g/l $Na_2EDTA.2H_2O$ in water pH 8.3 is reached when diluted to 1x working solution (stored at room temperature)

Polyacrylamide gel.

15.1g/l Tris base, 72.0g/l glycine in double distilled water (stored at room temperature) pH 8.3 was reached when diluted 1x working solution.

30% Acrylamide /0.8 Bisacrylamide solution

90g Acrylamide, 2.4g bisacrylamide in 300ml sddw. Filtered and stored at 4°C.

5% Stacking gel solution (50ml).

8ml 30% Acrylamide / 0.8% Bisacrylamide 6.25ml Tris-HCl, pH 6.8, stored at 4°C

12.5% Separating (Resolving) gel solution (30ml).

15.0ml 30% Acrylamide/0.8% Bisacrylamide solution, 3.75ml Tris HCl (pH 8.8), 30mg Ammonium Persulphate, 20µl TEMED.

Gel loading

Buffer

5xOrange G.

20% (w/v) Ficoll, 25mM EDTA, 2.5% (w/v) orange G. Stored at room temperature.

Silver staining solution.

Fixative

10% EtOH: 0.5% glacial acetic acid. Stored at room temperature.

Staining solution.

0.984g $AgNO_3$ in 500ml ddw (11mM). stored in the dark at room temperature

Developing solution 15ml 5M NaOH, 0.8ml Formalin made up to 100ml with sddw (Freshly prepared).

Quenching solution 10% glacial acetic acid. Stored at room temperature.

Solutions for ELISA.

Coating buffer (Bicarbonate Buffer pH 9.8)

Na_2CO_3	1.5g
$NaHCO_3$	2.93g
NaN_3	0.20g

Distilled water to 1000ml (stored at 4°C.)

Phosphate buffer saline- Tween (washing buffer pH 7.4)

NaCl	8.0g
KH_2PO_4	0.2g
$NaHPO_4\ 12H_2O$	2.9g
KCl	0.2g
NaN_3	0.2g
Tween-20	0.5g

Distilled water to 1000ml (stored at 4°C.)

Appendix 2

A graph of Log molecular weight against distance(s)/ cm travelled by the DNA fragments.