GENETIC DIVERSITY OF *PLASMODIUM FALCIPARUM* AND *PLASMODIUM VIVAX* ISOLATES IN DIFFERENT ENDEMIC ZONES OF ETHIOPIA

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GENETIC DIVERSITY OF *PLASMODIUM FALCIPARUM* AND *PLASMODIUM VIVAX* ISOLATES IN DIFFERENT ENDEMIC ZONES OF ETHIOPIA

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Table of Contents

Acknowledgements ..... **i**

List of Figures ..... **iv**

List of Tables ..... **v**

List of Appendices ..... **vi**

List of Abbreviations ..... **vii**

Abstract ..... **ix**

1. Introduction **1**

1.1.Parasite Biology 1

1.2. Malaria Symptoms 3

1.3. Epidemiology of Malaria 4

1.3.1. Global Burden 4

1.3.2. Malaria Situation in Ethiopia 5

1.3.3. The Economic Burden of Malaria 8

1.4. Genetic Structure of Plasmodium Spp 8

1.4.1. The Genome of Plasmodium Spp 8

1.4.2. Genetic Recombination 9

1.4.3. Genetic diversity in *P. falciparum* 10

1.4.4. Factors associated with multiclonal *P. falciparum* infections 12

1.4.5. Allelic/Antigenic Variation as Immune Evasion Strategy 13

1.4.6. Markers of Genotyping in *P. falciparum* 16

1.4.7. Genetic Diversity in *P. vivax* 17

1.5. Significance and Justification of the Study 19

2. Objectives of the Study **22**

2.1. General Objective 22

2.2. Specific Objectives 22

3. Materials and Methods **23**

3.1 Study Areas 23

3.1.1. Arbaminch 23

3.1.2. Zeway 24

3.1.3. Derra....24
3.1.4. Burie....25
3.2. Study Participants....25
3.3. Laboratory Methods....26
3.3.1. Blood Sample Collection....26
3.3.2. Giemsa Staining....27
3.3.3. Handling and Transportation of the Samples....27
3.3.4. Microscopy....28
3.3.5. DNA extraction from blood spotted filter paper....28
3.3.6. Genotyping of *P. falciparum* by Family specific nested PCR....29
3.3.7. Genotyping *P. vivax* by PCR-RFLP....31
3.5. Ethical considerations....33
3.6. Data Management, Interpretation and Analysis....34
4. Results....36
4.1. Study Population....36
4.2. Clinical Parameters of Malaria in the Study Population....38
4.3. Parasite Prevalence....39
4.4. Parasite Genotyping....41
4.4.1 Genotyping *P. falciparum*....41
4.4.2. Genotyping *P. vivax*....47
5. Discussion....49
6. Conclusions....56
7. Recommendations....57
References....58
Appendix A....67
Appendix B....69
Appendix C....70

List of Figures

Figure 1: The life cycle of malaria parasites. 2
Figure 2: Global Distribution of malaria transmission..... 5
Figure 4: Schematic representation of the sequence variations and deletions of the merozoite surface protein 3 (MSP3) gene of *Plasmodium vivax* among field isolates.....18
Figure 5: Map of Ethiopia showing locations of the four study sites..... 23
Figure 6: Malaria parasite species prevalence by microscopy and PCR in the four study sites, in 2004/2005.....38
Figure 7: Mean parasite density of *P. falciparum* and *P. vivax* in different age groups.....40
Figure 8: Age distribution of multiplicity of infection using MSP-2 gene of *P. falciparum*... 41
Figure 9: A gel picture of *P. falciparum* MSP 2 gene Nested PCR amplified product.....42
Figure 10: Allelic Frequency of FC27 allelic family of MSP-2 gene in *P. falciparum*..... 43
Figure 11: Allelic frequency of IC-1 allelic family of MSP-2 gene in *P. falciparum*.....44
Figure 12: Undigested PCR products of MSP 3 gene of *P. vivax*..... 47
Figure 13: PCR-RFLP products of *Hha I* digestion of MSP-3α gene of *P. vivax*.....48

List of Tables

Table 1: Primer sequences used for amplification of MSP-2 gene in *P. falciparum*……29

Table 2: Demographic information about the study participants from Arbaminch, Burie, Derra, and Zeway, in 2004/2005……...37

Table 3: Sensitivity and Specificity of PCR and Microscopy……..41

Table 4: Age group distribution of mean number of genotypes in the four study sites……45

Table 5: Association of multiple infections and clinical parameters……....46

List of Appendices

Appendix A: Case record form..... 67
Appendix B: Consent form – English version..... 69
Appendix C: Consent form – Amharic version..... 70

List of Abbreviations

AHRI	Armauer Hansen Research Institute
ALERT	All Africa Leprosy and Tuberculosis Rehabilitation, Research and Training Center
AMA	apical membrane antigen
bp	base pair
CSP	circumsporozoite protein
CTL	cytotoxic T lymphocyte
DNA	deoxyribonucleic acid
dNTP	nucleotide triphosphate
EBA	erythrocyte binding antigen
EDTA	Ethylene Diamine Tetra acetic acid
EIR	entomological inoculation rate
MOH	Ministry of Health
GLURP	glutamate rich protein
HIV	human immunodeficiency virus
IgG	Immunoglobulin G
KDa	kilo Dalton
Mb	mega base
MHC	major histocompatability complex
MOI	multiplicity of infection
MSP	Merozoite Surface Protein
PCR	polymerase chain reaction
PF	*Plasmodium falciparum*
PfEMP	*Plasmodium falciparum* erythrocyte membrane protein
PFG	pulsed field gel
PV	*Plasmodium vivax*
PvCSP	*Plasmodium vivax* circumsporozoite protein
PvMSP	*Plasmodium vivax* merozoite surface protein
RBC	red blood cells
RFLP	restriction fragment length polymorphism

SNP	Single nucleotide polymorphism
TAE	Tris acetate Ethylene Diamine Tetra-acetic acid
WBC	white blood cell
WFP	Whatman filter paper
WHO	World Health Organization

Abstract

Genetic typing of the parasites has been used as a marker for determining the level of transmission, level of acquired immune response and for analyzing the relationship between infection and pathogenesis of malaria. The present study is aimed at describing the prevalent parasite population dynamics of Ethiopian isolates in relation to age related protection and parasite pathogencity in different geographical settings. For this purpose, finger prick blood samples were collected on filter paper and slides from microscopically confirmed malaria patients (age > 3 months) attending health clinics in Arbaminch, Burie, Derra, and Zeway. Then DNA was extracted by chelex extraction method and used for PCR amplification: family-specific nested PCR of MSP-2 gene for genotyping *P. falciparum* and PCR-RFLP analysis of MSP-3α gene was used to genotype *P.vivax*. The findings of the study showed that mean number of genotypes for *P. falciparum* was 2.34 with a range of 1 to 6 and 78.2% of the isolates were multiclonal majority of them carrying double infection. Thirty-seven alleles were detected in FC27 (17) and IC-1 (20) allelic families. No significant associations found between age and multiplicity of infection while the difference was significant in different geographical areas. Fifteen different genotypes of *P. vivax* were found and 7.2% of the isolates were multiclonal. Based on the results of the study it was concluded that Ethiopian isolates *P. vivax* and *P. falciparum* are highly diverse with a pattern similar to other countries with the same level of transmission. Further nation wide investigation is recommended to better understand polymorphism of malaria parasites in relation to acquired immune response and pathogenicity.

1. Introduction

1.1. Parasite Biology

The microorganisms causing malaria are commonly referred to as malaria parasites; This term is restricted to the family *Plasmodidae* within the order *Coccidiidae*, suborder *Hemosporidiidae,* which comprises various parasites found in the blood of reptiles, birds and mammals (Schmidt and Roberts, 1983). Four species of the genus *Plasmodium* cause nearly all malarial infections in humans (although rare infections involve species normally affecting other primates). These are *P. falciparum, P. vivax, P. ovale,* and *P. malariae.* However, almost all deaths are caused by falciparum malaria while the other species can cause severe morbidity.

The life cycle of all species of human malaria parasites is essentially the same. Infection begins when a female anophelene mosquito inoculates plasmodial sporozoites from its salivary gland during a blood meal. These microscopic motile forms of malarial parasites are carried rapidly via the blood stream to the liver, where they invade hepatic parenchymal cells and begin a period of asexual reproduction. By this amplification process (known as pre-erythrocytic schizogony or merogony), a single sporozoite eventually may produce 10,000 to more than 30,000 daughter merozoites and then the swollen liver cells eventually burst, discharging motile merozoites into the blood stream (White and Breman, 2001). At this point the symptomatic stage of the infection begins. In *P. vivax* and *P. ovale* infections, the proportion of intrahepatic forms does not divide immediately but remains dormant for months to years before reproduction begins. The dormant forms or hypnozoites, are the cause of the relapses that characterize infection with these two species (Gilles and Warrell, 1993; Schmidt and Roberts, 1983; Bruce-Chwatt, 1980)

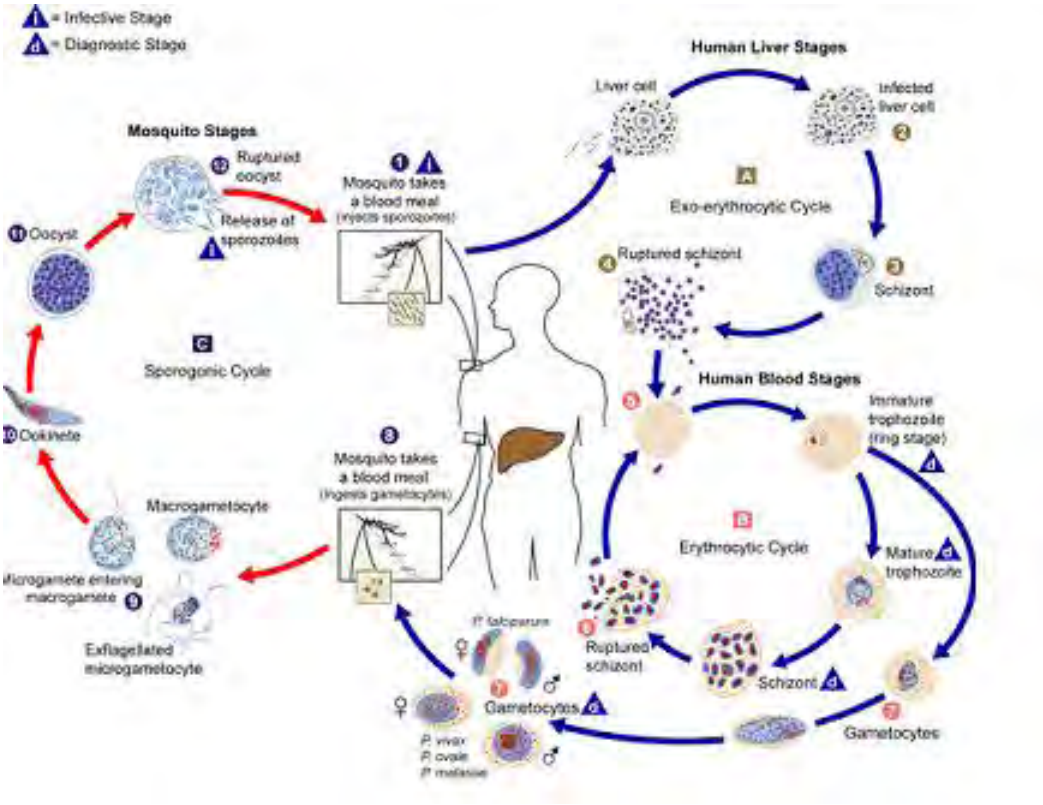


Figure 1The life cycle of malaria parasites. www.dpd.cdc.gov/dpdx

The malaria parasite life cycle involves two hosts. During a blood meal, a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the human host ❶. Sporozoites infect liver cells ❷ and mature into schizonts ❸, which rupture and release merozoites ❹. (Of note, in *P. vivax* and *P. ovale* a dormant stage [hypnozoites] can persist in the liver and cause relapses by invading the bloodstream weeks, or even years later.) After this initial replication in the liver (exo-erythrocytic schizogony A), the parasites undergo asexual multiplication in the erythrocytes (erythrocytic schizogony B). Merozoites infect red blood cells ❺. The ring stage trophozoites mature into schizonts, which rupture releasing merozoites ❻. Some parasites differentiate into sexual erythrocytic stages (gametocytes) ❼. Blood stage parasites are responsible for the clinical manifestations of the disease. The gametocytes, male (microgametocytes) and female (macrogametocytes), are ingested by an *Anopheles* mosquito during a blood meal ❽. The parasites' multiplication in the mosquito is known as the sporogonic cycle C. While in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes ❾. The zygotes in turn become motile and elongated (ookinetes) ❿which invade the midgut wall of the mosquito where they develop into oocysts ⓫. The oocysts grow, rupture, and release sporozoites ⓬, which make their way to the mosquito's salivary glands. Inoculation of the sporozoites into a new human host perpetuates the malaria life cycle ❶.

After entry into the blood stream, merozoites rapidly invade erythrocytes and become trophozoites. Attachment is mediated via a specific erythrocyte surface receptor. During the early stage of intraerythrocytic development, the small ring forms of the four parasitic species appear similar under microscopy. As the trophozoites enlarge, species specific characteristics become evident, pigment becomes visible, and the parasite assumes an irregular or ameboid shape. By the end of 48 hour intra-erthrocytic life cycle (72 hours for *P. malariae*), the parasite has consumed nearly all the hemoglobin and grown to occupy most of the red cell. Multiple nuclear divisions take place (merogony), and the red cell ruptures to release 6 to 30 daughter merozoites, each capable of invading a new red cell and repeating the cycle.

After a series of asexual cycles (*P. falciparum*) or immediately (P. *vivax* and *P.malariae*), some of the parasites develop into morphologically distinct long lived sexual forms (gametocytes) that are the transmission stage to the mosquito vector. After being ingested in the blood meal of a biting female anopheles mosquito, the male and female gametocytes form a zygote in the insect's mid gut. This zygote matures into an ookinete, which penetrates and encysts in the mosquito's gut wall. The resulting oocyt expands by asexual division until it bursts to liberate myriad motile sporozoites, which then migrate in the hemolymph to the salivary gland of the mosquito to await inoculation into another human host at the next feeding (White and Breman, 2001; Gilles and Warrell, 1993).

1.2. Malaria Symptoms

Generally the disease in human beings is caused by the direct effects of red cell invasion and destruction by the asexual parasite and the host's reaction to it. The incubation period is usually 10 to 20 days for *P. vivax,* 12 to 14 days for *P. falciparum,* and about 1 month for *P. malariae.* The malarial paroxysm (rigor) that coincides with the release of merozoites from ruptured red blood cells (RBCs) starts with malaise, abrupt chills and fever rising to 39 to 41° C (102 to 106° F), rapid pulse, and increasing headache and nausea. Next, fever falls and profuse sweating occurs over a period of 2 to 3 hours.

Manifestations common to all forms of malaria include anemia, jaundice, splenomegaly, hepatomegaly, and malarial paroxysm.

In *P. falciparum* malaria, infected red cells are known to adhere to uninfected red cells to form rosettes. The process of cytoadherence and rossetting are central to the pathogenesis of falciparum malaria. They result in sequestration of red cells containing mature forms of the parasite in vital organs, particularly in brain, where they interfere with microcirculatory blood flow and metabolism. Severe malaria also is associated with reduced deformability of the uninfected erythrocytes, which compromises the passage through the partially obstructed capillaries and venules and shortens red cell survival (Waliker *et al*, 1998). Placental involvement may lead to spontaneous abortions, stillbirths, or rarely congenital infection (Gilles and Warrell, 1993).

P. vivax and *P. ovale* rarely compromise the function of vital organs. Mortality is rare and is mostly due to splenic rupture or uncontrolled hyperparasitemia in asplenic persons. *P. malariae* infections often cause no acute symptoms, but low-level parasitemia may persist for decades and lead to immune complex-mediated nephritis or nephrosis or to "big spleen disease" (White and Breman, 2001).

1.3. Epidemiology of Malaria

1.3.1. Global Burden

Malaria occurs throughout most of the tropical regions of the world. *P. falciparum* dominates in Africa, New Guinea, and Haiti while 80 to 90 % of *P. vivax* occurs in Central America and Indian sub- continent (Mendis *et al*, 2002). The prevalence of these two species is approximately equal in South America and Eastern Asia. The remaining two species are much less common than the rest. Although the repeated attacks of *P. vivax* through childhood and adult life are only rarely directly lethal, they can have major deleterious effects on personal well-being, growth, development, and on the economic performance at the individual, family, community, and national levels.

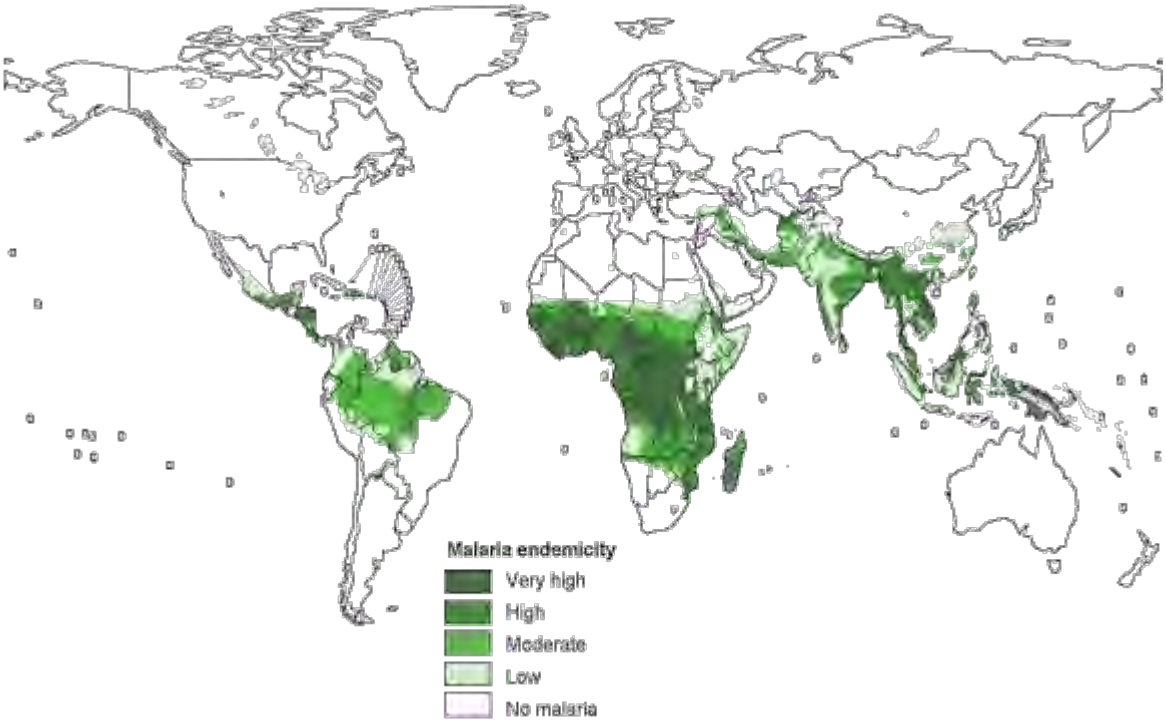


Figure 2: Global Distribution of malaria transmission, 2003

Source: (WHO, 2005)

Malaria is the most important parasitic disease affecting 300 to 500 million people in 90 countries in the world, causing 1.5 to 2.7 million deaths each year (Asante *et al*, 2004). It particularly remains a major public health problem in the tropics, especially in sub-Saharan Africa where the climate favors the growth of vector insect populations. Thus, 80% of malaria morbidity and 95% of malaria mortality worldwide occurs in these regions (WHO, 2005). The burden of disease is highest in children under five years of age. Most of the mortality due to malaria is attributed to lack of access to health care, life saving drugs and delay in seeking treatment.

1.3.2. Malaria Situation in Ethiopia

Malaria is prevalent in 75% of the surface area of Ethiopia, putting over 45 million people at risk and affecting 4 to 5 million people annually. It is one of the country's top ranking

communicable diseases and was the leading cause of mortality and morbidity in the year 2003/2004. In that year, malaria accounted for 15.5% of all out patient consultations, 20.4% of all inpatient admissions and also remains a major cause of mortality with proportional mortality rate of 27% in health care facilities. On average, about 700,000 people with positive blood films for malaria and 5 million clinical cases were treated in the year (MOH, 2005). The proportional morbidity among children under five years of age was 10 to 20% (MOH, 2000b).

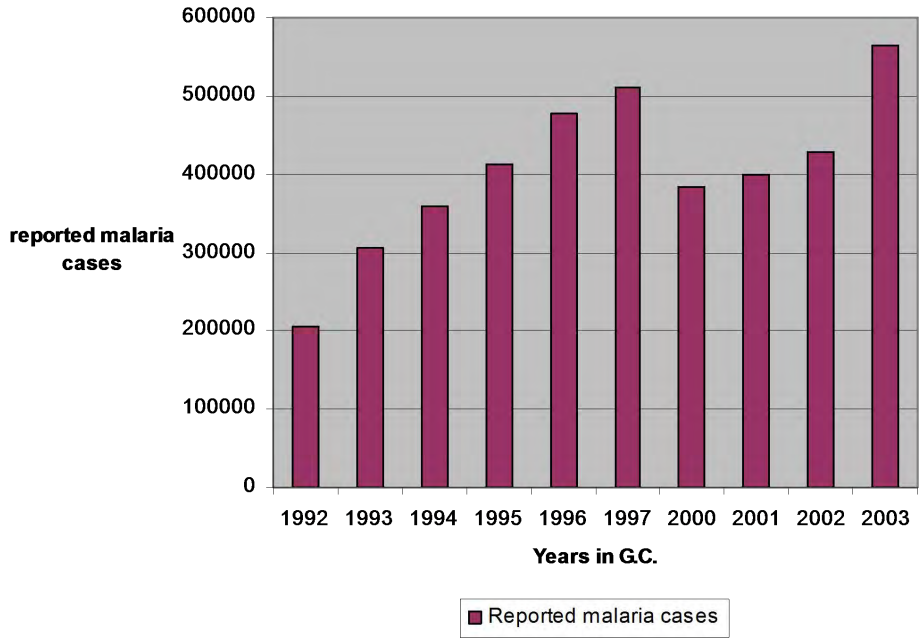


Figure 3: Annual microscopy confirmed malaria cases

Source: (WHO, 2005)

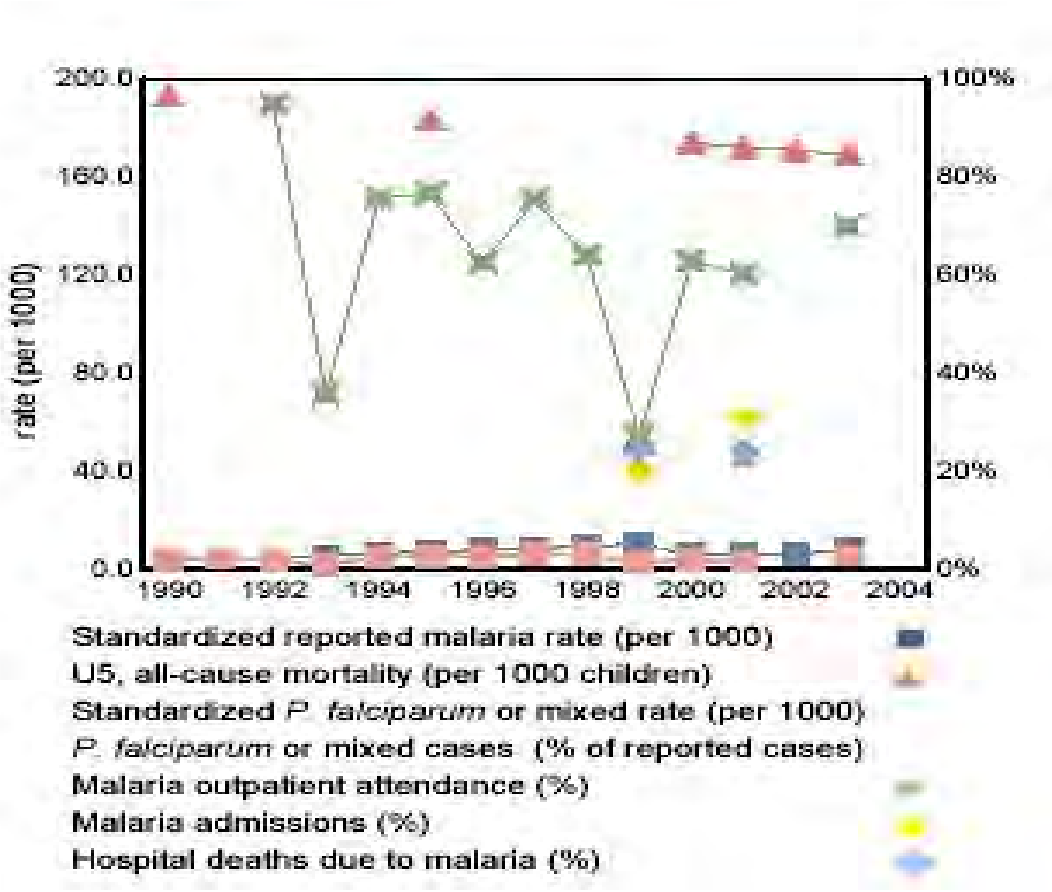


Figure 3: Malaria situation in Ethiopia

Source: (WHO, 2005)

All four human malaria parasite species occur in Ethiopia. However, *P. falciparum* is dominant accounting for 60 to 70% of the cases followed by *P. vivax*, which is responsible for 30 to 40% of cases. *P. malariae* and *P. ovalae* are rare, accounting for less than 1% of the cases (MOH, 2000a; MOH, 2005). In Ethiopia, altitude and climate (rainfall and temperature) are the most significant determinants for malaria transmission. Generally, areas lying below 2,000 meters altitude are major malaria transmission sites, as they favor the growth of relevant mosquito vector populations. Since the transmission intensity is different at different altitudes, altitude is expected to cause slight variation in the population genetics of parasites. The pattern of transmission is mainly seasonal. Transmission seasons occur following '*kiremt'* and '*belg'* rainy seasons. The major transmission season comes just after *'kiremt'* rainy season, which occurs between September and December and covers almost every part of the country. The major vector

of malaria is *Anopheles arabiensis* which prefers fresh, sunlit, small water collections for its breeding. *A. pharoniesis, A. funestes* and *A. nili* are secondary vectors.

1.3.3. The Economic Burden of Malaria

Malaria as a disease is not only a public health problem but also a major threat to economic development. Malaria mortality and morbidity slows economic growth by reducing the capacity, and efficiency of the labour force and by the direct effect of costs related to transportation to health service facility, consultation fee, laboratory test fee, and more importantly, drug cost. In a study in Ghana, it was shown that the cost of illness reached up to 17.4 USD per episode per household (Asante *et al*, 2004). The economic loss due to malaria is very high, with an annual loss of growth estimated at 1.3% per year and approximately 12 billion US dollars lost every year due to malaria in Africa (WHO, 2005). In Ethiopia, due to high malaria transmission, fertile lowlands and major river valleys have not been fully inhabited and developed. As a result, highland areas become over populated, ecologically degraded and less productive, leading to famine and poverty (MOH, 2000b).

Today, malaria is a heavy burden on tropical communities, and a threat to non-endemic countries and travelers. Even though the disease in countries such as the USA is not as prevalent as it is in sub-Saharan Africa, resurgence of malaria there is evident now due to a variety of causes, including increased population movement and global temperature change (Shell, 1997; Gardner *et al*, 1998), indicating a continual danger of malaria occurrence or re-emergence in non-malarious countries.

1.4. Genetic Structure of Plasmodium Spp.

1.4.1. The Genome of Plasmodium Spp.

Plasmodium is a eukaryote whose nuclear genome is organized into discrete chromosomes. Application of the Pulsed Field Gradient (PFG) gel electrophoresis method to *P. falciparum* has revealed the number of chromosomes to be 14 at first, ranging in size from

650Kb to 3.4Mb (Van der Ploeg *et al*, 1985; Kemp *et al*, 1985). Homologous chromosomes varied considerably in their size in different wild isolates of *P. falciparum* (Kemp *et al*, 1985). Malaria parasites possess two extranuclear DNA elements. One is a linear mitochondrial DNA element with tandemly repeated 6Kb sequences and a very limited number of mitochondrial genes. The second element is a 35Kb circular DNA molecule within the apicoplast that codes for genes associated with plastid genome (Wilson and Williamson, 1997; Gardner *et al*, 2002). *P. falciparum* genome consists of A/T rich (80%) 23 Mb DNA containing almost 5,300 genes (Gardner *et al*, 2002; Meyer *et al*, 2002). The *P. vivax* genome, unlike *P. falciparum*, has a much lower A/T content (55%) (Cui *et al,* 2003).

1.4.2. Genetic Recombination

All genetic variation originates with gene mutation, but once it has arisen, recombination is the principal mechanism for generating forms of an organism with novel genotypes. In eukaryotes, recombination occurs principally at meiosis and much more rarely at mitosis. A study has shown that chromosomal deletions and translocations which may involve recombination events can occur during prolonged asexual culture of *P. falciparum but* no information is available on such events in natural infections (Walliker *et al*, 1998).

In general, meiosis allows an independent assortment of genes on different chromosomes, crossing over events between linked genes on the same chromosome and intragenic recombination. The malaria parasite reproduces asexually and is haploid for most of its life cycle in the vertebrate hosts. However it produces gametes and undergoes fertilization, zygote formation in mosquitoes, with the only diploid stage being the zygote formed by the union of gametes in the mosquito (Walliker *et al*, 1998; Bruce-Chwatt, 1980). During fertilization in the mosquito gut, combination between genetically identical gametes, originating from a single clone produces zygotes homozygous at all loci. Here meiotic division is not expected to have major genetic effects because both alleles of all genes are identical although some errors in replication and crossing over may produce new alleles. If gametocytes of more than one clone are taken up, crossing over events result in

heterozygotes. Here, meiotic recombination inevitably leads to the production of recombinant haploid forms.

In the asexual phase of its life cycle, because it remains haploid for most of its life cycle in the vertebrate host, frequent fertilization may offer an important evolutionary advantage. High recombination rates ensures a rich variety of haploid states that maintain diversity in parasite population. In wild populations, the rate of genetic assortment and recombination in a population of Plasmodium parasites depends on the frequency at which vector mosquitoes acquire blood meal containing gametocytes of more than one genotype. It was proposed that at least some multiple clone infections result from multiple clone inoculations from a single mosquito carrying different recombinant parasites (Conway et *al*, 1991) rather than inoculation by more than one mosquito (Arnot, 1998).

1.4.3. Genetic diversity in *P. falciparum*

P. falciparum shows diversity in variant forms of antigens, proteins, enzymes, in the sequence of many genes and responses to drugs (Creasy *et al*, 1990; Conway and McBride, 1991; Volkman *et al*, 2001, Feng *et al*, 2003). Studies are revealing that some variants of these characters occur at different frequencies in different geographical areas (Walliker, 1985; Creasy *et al*, 1990).

P. falciparum infections containing more than one genotype were first identified *in vitro* (Rosario, 1981) by demonstrating heterogeneity of parasites with respect to electrophoretic forms of glucose phosphate isomerase (GPI). After that, isoenzyme analysis, monoclonal antibody immunoflouresence, 2-dimensional-gel electrophoresis, drug susceptibility assay, hybridization, Polymerase Chain Reaction (PCR), Restriction Fragment Length Polymorphism (RFLP) analysis and direct sequencing of PCR products were used to identify multiple-clone *Plasmodium* infections (Thaithong *et al,* 1984;

Conway *et al*, 1991; Kain *et al*, 1991; Roper *et al*, 1996; Adagu and Warhurst, 1999; Felger *et al*, 1994).

The inherent variability of *P. falciparum* provides multiple effective immune evasion and drug resistance mechanisms for the parasite. Many studies on the malaria parasites polymorphism have focused on variants exhibiting mutations that lead to amino acid substitutions (non-synonymous mutations) that are most likely subjected to selection of immunogenic proteins and drug resistance phenotypes (Coopel *et al*, 2004). Genes encoding proteins that are expressed on the surface of sporozoites and merozoites, in general, are more variable than genes expressed during the sexual stages (Niederwieser *et al*, 2001).

Single nucleotide polymorphisms (SNP) contribute largely to the variability. Alignment of sequences based on information obtained from multiple geographical locations have shown that the synonymous mutations are scarce in most of the parasite genes (Meyer *et al*, 2002). Other studies, that compared different laboratory isolates from different geographical locations, revealed that genetic variation was concentrated in genes encoding membrane proteins such as MSP-2, but not randomly distributed across coding genes (Coopel *et al*, 2004; Creasy *et al*, 1991). This suggests selection for variation in these genes, with the host immune response being the most likely selection pressure. Thus, these proteins create problems in targeting them in the design of vaccine against the parasite (Escalante *et al*, 1998). Non-synonymous SNPs are found at high frequencies at high selective pressure, in particular those genes encoding proteins linked to immune evasion or drug resistance.

Genes where polymorphism has arisen through intragenic recombination in repetitive segments are characterized by repeated motifs with length variability differing between strains (Waliker *et al*, 1987). Among these genes are those encoding the *P. falciparum* circumsporozoite protein, a glutamate-rich protein, two merozoite surface proteins (MSP-1 and MSP-2), and the apical membrane antigen (AMA) (Meyer *et al*, 2002).

1.4.4. Factors associated with multiclonal *P. falciparum* infections

The genetic diversity of local *Plasmodium* populations may be determined by the number of parasite clones in infected human individuals. In endemic areas, the number of clones of malaria parasites co-infecting a single host can be a useful indicator of the level of transmission and/or the immune status of the host (Owusu-Agyei *et al*, 2002). *P. falciparum* infections have been found to be genetically diverse in all except unusual situations (such as in an epidemic in an isolated population), and most infections in endemic transmission zones carry more than one parasite clone (Arnot, 1998). Diverse multiclonal infections are found to be common in areas where malaria transmission is seasonal and unstable as it is in areas of higher transmission (Conway and McBride, 1991; Arnot, 1998, Babiker *et al*, 1998). Although Entmological Innoculation Rate (EIR) is not an efficient measure for malaria transmission, it was found that increasing number of clones is associated with progressively increased rate of EIR (Arnot, 1998).

Genetic typing of parasites has also been used as a marker for analyzing the relationship between infection and pathogenesis of malaria. The conversion of individuals from a state of asymptomatic *P. falciparum* infection to clinical malaria has been correlated with changes in the composition of the parasite population (Contamin *et al*, 1996). Association has been reported between more virulent infections and certain *P. falciparum* genotypes (Engelbricht *et al*, 1995). In contrast, the number of clones per infection appeared to have no relevance to the development of severe disease (Conway *et al,* 1998). The number of genetically distinct parasite clones in individual infections has been found to vary between asymptomatic and clinically ill patients, with a trend towards increased number of clones in asymptomatic persons (Contamin *et al*, 1996; al Yaman *et al*, 1997). However, the tendency to detect more mixed infections in clinical cases emerged in a study of hypoendemic and unstable malaria in Sudan (Arnot, 1998). This shows that the issue of multiple clone infection versus clinical disease development may vary among areas of different levels of transmission.

Significant differences in the percentage of mixed infections that may reflect intensities of infection, transmission patterns, human and vector distribution, and the population dynamics of the parasite was documented in a study in three different countries (Creasy *et al,* 1990). It was concluded that there are geographical variations in the frequencies with which many variants occur. Therefore, it is relevant to study the extent of variation which may occur in different communities and geographical localities.

Multiplicity of infection was found to be age dependent (Owusw-Agyei *et al*, 2002) in areas of higher transmission with a trend of highest values in children. In areas with low transmission, little or no influence of age on infection complexity and allelic distribution of *P. falciparum* was detected (Conway *et al*, 1991). During clinical malaria episodes, older children generally have been observed to have lower measurable clonal multiplicities than equivalent groups of asymptomatically infected children (Farnert *et al*, 2001). In situations where higher multiplicities, have been reported in older children, this has been correlated with the reduction in the risk of contracting clinical malaria (al Yaman *et al*, 1997). Furthermore, higher number of clones is not associated with protection in infants where those experiencing a febrile episode actually had significantly higher mean multiplicities than asymptomatic equivalents in a higher transmission site. Also, higher clone numbers were not associated with protection in areas of unstable and low transmission (Ofusu-Okyere *et al*, 2001).

All these contradicting findings indicate the lack of determination of the relationship of multiple clone *P. falciparum* infections and age, protection, level of transmission and pathogenesis of disease.

1.4.5. Allelic/Antigenic Variation as Immune Evasion Strategy

Allelic diversity in malaria parasites is widely assumed to be a mechanism for immune evasion. Among surface proteins, diversity arises from multiple point mutations or variations

in the numbers, lengths, and sequences of amino acid repeats. The AT-rich nature of the *Plasmodium falciparum* genome may facilitate the generation of new variants. The maintenance of variants in the population implies that they have a selective advantage (e.g., evasion of preexisting immune responses) or that the mutations are selectively neutral and that population diversification occurs randomly (Franks *et al*, 2003).

The ability of *P. falciparum* to re-infect previously exposed individuals is due to the existence of phenotypically variant strains of the parasite in an endemic area. The epidemiology of malaria is heavily dependent upon this variance (Hommel, 1985). Clinical studies have shown that immunity to malaria requires repeated infections and is slow to develop, and that children under ten years of age are the most susceptible to grave illness and fatality. This pathology is explained by the diversity of *P. falciparum* strains and the variant allelic forms of parasite proteins that they produce. Cerebral malaria, a fatal form of the disease, is caused by the aggregation of infected red blood cells adhering to and blocking capillaries in the brain. Adults who have been exposed to variant strains of *P. falciparum* are able to produce sufficient humoral response to parasite proteins involved in parasite cytoadherence. Children, however, have not been immunologically exposed to this diverse body of parasite proteins. Their inability to mount a strong immune response to variant strains can result in fatality (Hommel, 1985; Conway *et al*, 2000).

Antigenic diversity at a level of the individual parasite occurs during the course of infection of a given individual. Variation occurs on the surface antigens of infected erythrocytes during the erythrocytic schizogony phase of the parasite life cycle. The sequestration of malaria parasites in human red blood cells during this phase of the cycle poses an extremely complex method for evasion of host immunity. First, infected RBCs do not induce cytotoxic T-cell lymphocyte (CTL) response due to their lack of MHC expression. Second, parasite-derived particles exposed on the surface of the cell are highly variable, leading to the inability of the immune response to produce adequate memory to the antigens. These molecules are associated with pfEMP-1 proteins and undergo high clonal variation (Conway *et al*, 2000; Boland and Berzins, 2000). This variation is immunologically important as it results in the host's inability to produce appropriate antibodies within a limited time frame of erythrocyte

infection. The specific mechanism of the gene expression regulation has not yet been determined.

In some genes (e.g., the circumsporozoite surface protein [*csp*] gene), point mutations cluster in areas immunodominant for T cells and can lead to loss of epitope recognition or antagonism of T-cell responses although it is not clear that either of these leads to the avoidance of protective immunity (Day and Marsh, 1991). Similarly, monospecific antibodies differentiate between allelic variants of merozoite surface proteins, but the human antibody response is commonly directed at conserved or semi-conserved epitopes and direct evidence for the evasion of antibody-dependent immunity is lacking (Boland and Berzins, 2000).

Comparison of the frequencies of non-synonymous-to-synonymous mutations for *P. falciparum* genes provides evidence of diversifying selection in *msp-1*, which encodes merozoite surface protein-1, but the data are less clear for *MSP-2*, which encodes merozoite surface protein-2, and the significance of the findings is uncertain, given the limitations of the method (Franks *et al*, 2003). Comparison of intra- and interpopulation variances in allele frequencies provides evidence for selection on a polymorphic region of *msp-1* and dimorphic regions of *MSP-2* but there are no data for polymorphic regions of *MSP-2*. Comparison of inter- and intraspecific levels of variation suggests balancing selection on the erythrocyte-binding antigen (EBA-175) and the apical membrane antigen (AMA-1). However, except for *msp-1*, for which selection may be mediated by strain-specific antibodies, the nature of the selective forces is not clear (Franks *et al*, 2003; Conway *et al*, 2000).

Analysis of responses to polymorphic sequences is difficult, as it is rarely known with which parasite genotypes an individual has been infected and to which sequences he or she might be expected to have made antibodies. Nevertheless, sera from a high proportion of both children and adults do recognize recombinant proteins representing individual polymorphic sequences, indicating either that these sequences are common in the parasite population or that they contain epitopes that cross-react with other sequences. There is good evidence that anti-MSP-2 antibodies contribute to protective immunity (Franks *et al*, 2003). In independent

epidemiological studies, immunoglobulin G3 (IgG3) antibodies have been associated with resistance to clinical malaria, and anti-MSP-2 antibodies have inhibited the growth of parasites *in vitro*, and in a recent human trial, immunization with the 3D7 variant of MSP-2 prevented re-infection with parasites carrying alleles of the same family (IC-1) (Franks *et al*, 2003).

1.4.6. Markers of Genotyping in *P. falciparum*

The most commonly used markers for genotyping of *P. falciparum* populations are regions of genes coding for three surface antigens: the merozoite surface protein 1 (MSP 1), MSP 2, and glutamate rich proteins (GLURP) (Farnert *et al*, 2001; Cui *et al*, 2003). These have been considered suitable markers since they are single locus genes which exhibit greater polymorphism both in length and sequence. The length variation of these genes is primarily due to randomly repeated sequences and alleles that can be readily distinguished following electrophoresis of PCR products (Owusw-Agyei *et al*, 2002; Escalante *et al*, 1998). The choice of marker genes for genotyping of *P. falciparum* infections will obviously depend on the specific research questions to be asked. Although multiple locus analysis inevitably improves the estimated multiplicity of infection, the MSP-2 locus was generally found to be more informative than others (Farnert *et al*, 2001; Owusw-Agyei *et al*, 2002; Escalante *et al*, 1998).

MSP-2 is a membrane protein located on the merozoite and has a molecular weight of 45 to 54kDa. Like many other malaria surface antigens, MSP-2 also displays many repetitive amino acid sequences. It consists of N- and C- terminal regions with 43 and 74 residues respectively and a central variable segment made up of repetitive and non-repetitive segments (Rich *et al*, 2000; Escalante *et al*, 1998). These tandem repeats may stimulate T-independent B-cell responses that are potentially defective in that they do not generate memory B-cells or somatic hypermutation leading to antibody affinity maturation (Tonon, *et al*, 2004).

MSP-2 gene is the most polymorphic locus yet described for *P. falciparum*, with over 170 alleles sequenced to date (Franks *et al*, 2003). It has been considered the most informative marker of clonality and has been genotyped in many studies to assess the degree of multiple clone infection. MSP-2 alleles are divided into two families. These are IC-like and FC27-like based on dimorphic sequences internal to the conserved N- and C- termini and named after the isolates in which they were first identified. The FC27 family is characterized by coding at least one copy of a 32 amino acid sequence and a variable number of a 12 amino acid repeats; the IC-1/3D7 family codes tandem amino acid repeats of 4-10 amino acids in length (Rich *et al*, 2000; Meyer *et al,* 2002).

Point mutations occur in all regions of the gene (including conserved and dimorphic sequences), and variations in sequence, length and number of amino acid repeats occur in the polymorphic region. Most diversity in MSP-2 occurs in its central repeats (block 3), that are flanked by block 2 and 4 and conserved blocks 1 and 5. The central variable region even differs between and within the two allelic families (Tonon *et al*, 2004). Seroepidemiological studies indicate that the conserved N- and C- termini of the molecule are poorly recognized by immune serum but that the dimorphic sequences are strongly recognized by most adult immune sera (Meyer *et al,* 2002).

1.4.7. Genetic Diversity in *P. vivax*

In studies on the population structure of *P. vivax* considerable information is lacking as compared to *P. falciparum*. Some surveys have been carried out on the allelic variations of two genes, the circumsporozoite gene PvCSP and the PvMSP1, which is the homologue of PfMSP1 (Bruce *et al*, 1998; Cui *et al* 2003; Leclerc *et al*, 2004). Analyses of these genes from different countries have revealed extensive heterogeneity within *P. vivax* populations.

Generally, heterogeneity of the PvCSP gene is not as extensive as that seen in the *P. falciparum* homologue. Due to the dimorphic nature of PvCSP gene, classifying field isolates

either into VK210 or VK247 genotype does not provide enough evidence for the genetic diversity of *P. vivax*. There is some evidence that the prevalence of the two major allelic forms of PvCSP (VK210 and VK247) may differ between different geographical populations (Walliker *et al*, 1998). In a study in Azerbaijan, only one allelic family (VK210) was prevalent with 24/36 isolates having an identical sequence (Leclerc *et al*, 2004). In Thailand, the VK210 was the dominant allele accounting for more than three fourth of the alleles, but almost all infections with VK247 were multiple infections (Cui *et al*, 2003). For PvMSP 1 gene, the variable region between interspecies conserved block 5 and 6 has been used to genotype from many different geographical regions and appeared to be more polymorphic than PvCSP (Cheng *et al*, 1993; Bruce *et al*, 1999; Leclerc *et al*, 2004).

The *P.vivax* MSP 3 gene family, with the individual single copy gene members designated as MSP 3α, MSP3β, and MSP3γ, is related to *P. falciparum* MSP3. Although there is only 30% overall identity between the 3 MSPs, their unifying molecular characteristics, like PfMSP 3 is a large central domain that is high in alanine content (20 to 30%) (Barnwell and Galinski, 1998). The calculated molecular mass of PvMSPs ranges from 75 to 105 KDa, and there is a high degree of diversity in natural isolates within their central domains (Barnwell and Galinski, 1998).

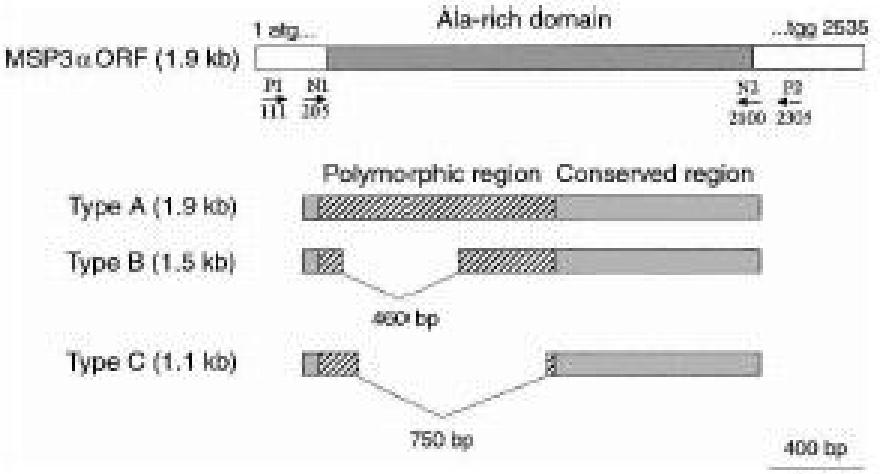


Figure 4: Schematic representation of the sequence variations and deletions of the merozoite surface protein 3 (MSP3) gene of *Plasmodium vivax* among field isolates

(Cui *et al*, 2003)

Pv MSP 3α, as it is introduced only recently in the study of genetic diversity of *P. vivax*, less information is available regarding the gene. But, it has been shown that it indicates the presence of great number of alleles. So far it was possible to detect 13 alleles by using this gene as a marker (Bruce *et al*, 1999; Cui *et al*, 2003).

The rate of multiclonal infections in *P. vivax* seems lower than for *P. falciparum*. In a large study in India, using three polymorphic enzymes 20% of the isolates were multiclonal with average multiplicity of infection (MOI) of 1.4 (Josh *et al*, 1997). There was no significant difference in the rate of multiclonal infection between times of high and low transmission seasons as well as in allelic frequencies over a period 8 years. This finding is in contrast to that of *P. falciparum* where there is a daily parasite population dynamic (Farnert *et al*, 1999).

For *P. vivax*, dimorphic PvCSP gene and PvMSP-1 genes have been widely used for genotyping. But these genetic markers are found to have either inefficient polymorphism, require complex methods or sequencing for the detection of polymorphism, or require a large volume of blood for serologic and biochemical methods (Bruce *et al*, 1999, Cui *et al*, 2003). So, there was a need for other molecular markers that are more suitable for rapid genotyping. One such marker is PvMSP3α because its polymorphism can be readily evaluated by PCR-RFLP and can be used to indicate a great number of alleles (Bruce *et al,* 1999; Cui *et a*l, 2003).

1.5. Significance and Justification of the Study

Understanding the population genetics of pathogens helps to implement control measures by enabling one to make predictions about parasites' ability to develop drug resistant forms and to circumvent vaccines by producing vaccine resistant forms. The ability of malaria parasites to adapt to a changing environment such as drug pressure and immune response makes the control effort very difficult. The study of genetic variation in malaria parasites has practical

significance for developing strategies to the control of the disease. The knowledge of their heterogeneity in different transmission levels contributes greatly the understanding of the dynamics of the disease, which in turn guides our decision in drug selection and disease control. Vaccines based on the highly polymorphic antigens may be confounded by allelic restriction of the host immune response. In response to drug pressure, a highly plastic genome may generate resistant mutants more easily than a monomorphic one. Additionally, the study of the distribution of genomic polymorphisms may provide information leading to the identification of genes associated with traits such as parasite development and drug resistance.

Studying genetic variations of malaria parasites in different communities gives clues about whether the parasite population is reproductively related or isolated from one another or whether gene flow occurs between them through migration of human or mosquito hosts. Understanding this subject is relevant to eventually predict how effective control measures can be implemented as far down as a single village level. Specifically, information on the existence of genes that confer resistance to drugs or vaccines in an area has a practical value in planning and implementing control measures.

Almost all of the studies on *P. vivax*, as we have seen so far, were conducted either in Asia or Latin America where *P. vivax* is the predominant malaria parasite species. However, such information on *P. vivax* genetic diversity is lacking in Africa as the most predominant species in the continent is *P. falciparum*. Ethiopia is unique in Africa in having both species, *P. falciparum* and *P. vivax*, with almost equivalent prevalence accounting 60 % and 40% respectively. By taking this as an advantage, the genetic structure of *P. vivax* isolates in Ethiopia was studied with the most suitable gene marker, i.e. PvMSP3α.

There are promising vaccine candidates on the horizon indicating that the time for the discovery of an effective vaccine may not be too far away. Genetic variability of Ethiopian isolates should be described in order for the country to benefit from ongoing efforts to produce suitable candidate vaccines for different geographical areas. Ultimately, mapping out the genetic diversity of *P. falciparum* and *P. vivax* infection in Ethiopia will assist the

Ministry of Health and Regional Health Bureaus in properly designing and implementing as improved malaria control program.

The genetic structure of malaria parasite isolates has not yet been determined (MOH, 2000a). In this study, it is hypothesized that *P. falciparum* and *P. vivax* isolates in Ethiopia, where malaria transmission is unstable and seasonal, show as much genetic polymorphism as exists in other African countries, where malaria transmission is stable and all year round.

2. Objectives of the Study

2.1. General Objective

- To characterize the genetic diversity of Ethiopian *P. falciparum and P.vivax* isolates at different transmission sites using PCR-based methods

2.2. Specific Objectives

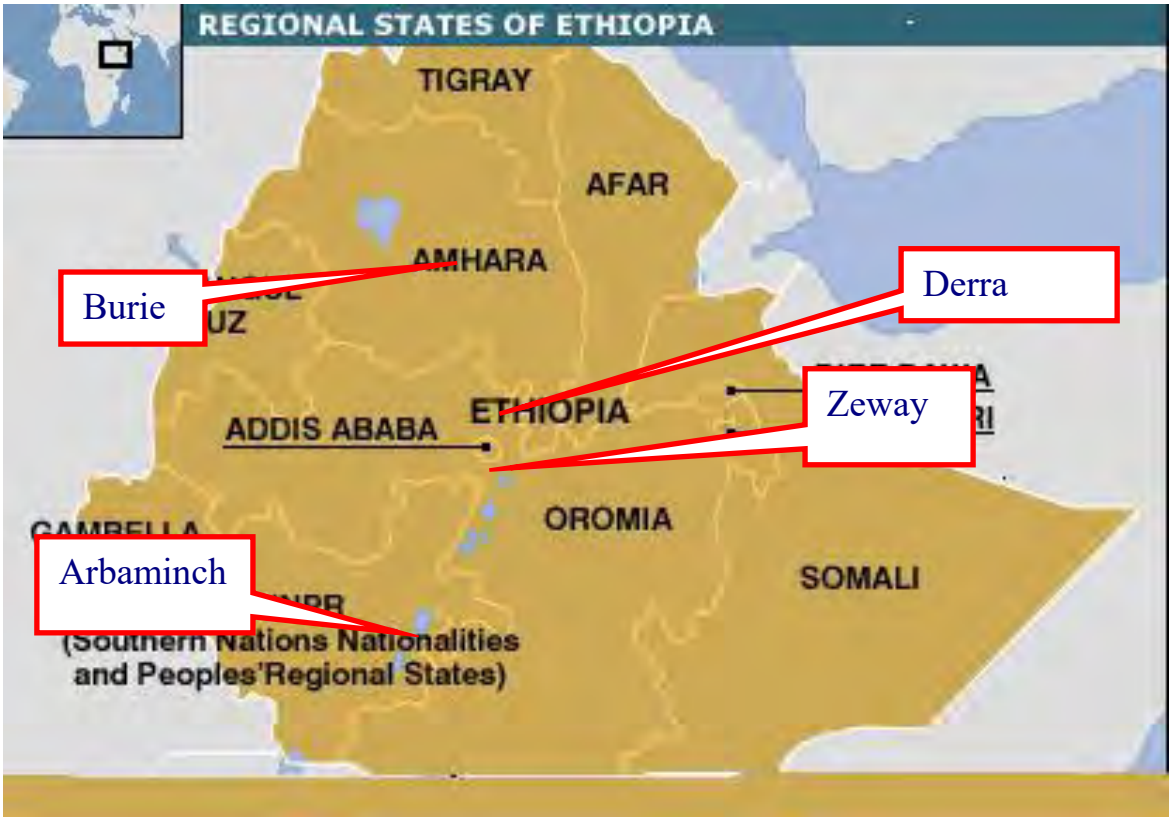
- To assess genetic variation of *P. falciparum* in MSP-2 gene loci by using family-specific nested PCR.
- To assess genetic variation of *P. vivax* isolates at MSP3α gene locus by using PCR-RFLP.
- To determine possible association between age and clonal-multiplicity of *P. falciparum* and *P. vivax* infection.
- To compare type of *P. falciparum* isolates from severe malaria cases with those types from uncomplicated malaria cases.
- To compare the magnitude of genetic variation of *P. falciparum* between different transmission foci.

3. Materials and Methods

3.1 Study Areas

Four different geographically distant sites of Ethiopia were selected as study areas: Arbaminch ($10^0$14’ N, $38^0$41’ E), Zeway (7^0 55’N, $38^0$43’ E), Derra ($6^0$02’ N, $37^0$41’ E) and Burie ($10^0$43’ N, $37^0$34’ E) (Fig. 5).

Figure 5: Map of Ethiopia showing locations of the four study sites



3.1.1. Arbaminch

Arbaminch Zuria District is located about 500kms south of Addis Ababa and is bordered partially by the rift valley lakes, namely *Abaya* and *Chamo*. It is found in Gamo Gofa zone, where 1,234,000 people live, in the Southern Peoples, Nations and Nationalities

Regional State (SNNPR). The rainfall pattern is somewhat different from other Ethiopian regions with its irregularity. A large proportion of the landmass in the district is lowland, where malaria occurs throughout the year, with some seasonal peaks following the rainy season. The economy of the people in the district depends on agriculture. Irrigation activities are common in governmental and private agricultural farms around Arbaminch town. Such conditions favor breeding of mosquito vectors. Malaria is the leading cause of morbidity in the area affecting all age groups.

The dominant malaria parasite species in the district is *Plasmodium falciparum*. Arbaminch Health Center, Arbaminch Hospital, Lante Health Center and Celo Sego Private Clinic in Shele rural town were health institutions from where study participants were obtained in this area.

Blood samples were collected in May 2004, and October 2004 and April 2005.

3.1.2. Zeway

Zeway is found in Oromiya Regional state, East Showa Zone and Judo Combolcha Adamitulu District. It is 163 kms south of Addis Ababa alongside of Lake Zeway. The area is mainly characterized by its low land feature. The rain fall pattern in the area is similar with other Ethiopian regions. Malaria is the principal cause of morbidity in the area affecting all age groups.

Both malaria parasite species are equally prevalent in the area. During intense malaria transmission following the rainy seasons, *P. falciparum* becomes dominant over *P. vivax*. The reverse is true during dry seasons.

Blood samples were collected in May 2004.

3.1.3. Derra

Derra district it found in North Showa Zone of Oromiya Regional State. The district has a total land area of about 1,528 square kilometers. Its capital town, Gundomesquel is situated at a distance of about 220kms north of Addis Ababa. Two agro-ecological zones

are found in the district, highland (*Dega*) and lowland (*Kola*) areas covering the district in equal proportion. The district receives a bimodal rainfall pattern, *kiremit* and *belg* (in highland areas) which are characterized by favoring malaria transmission, creating mosquito-breeding sites.

The dominant species in the district is *P. vivax.* Study participants were obtained from four health posts and one health center, namely, Gundomesquel Health Center, Salayish Health Post, Tuti Health Post, Karra Health Post, and Kasanesh Health Post. Malaria is the third cause of morbidity in the area.

Blood samples were collected between October and November 2004.

3.1.4. Burie

Burie district is one of twelve districts in East Gojam Zone in Amhara Regional State. It is situated 421 kms northwest of Addis Ababa. The total land area of the district is 73,468 km^2 with a population of 160,000. Midland '*Woynadega*' comprises 77% of the landmass. It gets bimodal rainfall, ranging from 1000 to 1,500mm in amount. Malaria transmission follows the two rainy seasons with frequent epidemics occurring in the area. A few years ago, malaria was not a major public health problem in the area. Now it is the first in the list of ten top diseases. The health coverage (21.9%) of the district is very much lower than the national coverage (60%) (Mr. Girma, personal communication, 2004; MOH report, 2005 Africa malaria day).

Both *P. falciparum* and *P. vivax* occur equivalently in the area. Study subjects were recruited from in Burie Health Center, which is the only health center in the district.

Blood samples were collected in September 2004.

3.2. Study Participants

Study sampling frame were patients who came to pediatric and adult outpatient departments of health care facilities at study sites with complaint for signs and symptoms of malaria. Study subjects were identified based on the laboratory results as diagnosed by

laboratory technicians at the local health institutions of four study sites. Only microscopy positive patients for *P. falciparum* and *P. vivax* were recruited. But in health posts where there were no microscopes, patients were recruited into the study based on microscopy reading of the principal investigator at AHRI. Because of the lack of power supply, microscopes could not be carried to health posts. After sample populations were identified, clinical and laboratory examination was done. Clinical case record forms were completed with study identification number. The calculated sample size was 246 at 95% confidence interval.

The inclusion criteria for the study were: greater than 3 months of age, microscopically positive for malaria and having informed consent from volunteer parents or guardians.

Patients who were microscopically positive for *P. falciparum* and present with at least one of the following symptoms and signs of severe malaria were considered as severe cases. These are severe anaemia (Hgb <5g/dL), unarousable coma (cerebral malaria), renal failure (low urine output), hypotension/shock, bleeding, convulsions, impaired consciousness, repeated vomiting and jaundice (Betemariam and Yayeh, 2002).

3.3. Laboratory Methods

3.3.1. Blood Sample Collection

Blood sample collection was carried out before appropriate treatment was given to the patients. A finger-prick was done by laboratory technician or the investigator and the blood directly spotted on to the labeled 3MM Whatman Filter Paper (WFP) (Wooden and Kyes, 1993). At the same time, thin and thick blood smears were prepared on two similarly labeled slides per patient for microscopy from the same prick. The thin and thick blood smears were prepared on the same slide side by side for each of two slides and allowed to air dry. The blood spots were allowed to air dry.

3.3.2. Giemsa Staining

The quality of Giemsa staining solution was checked before using directly for the samples. After blood film slides were allowed to air dry, they were stained with Giemsa stain according to the following protocols (Schilchtherle *et al*, 2000):

3.3.2.1. Giemsa staining of thick blood films:-A fresh 10% Giemsa solution in tap water was prepared and the air dried slides were immersed into staining jar for 10 minutes. The stained slides were rinsed by tap water and put in upright position to dry.

3.3.2.2. Giemsa staining of thin blood films: - Air dried thin film slides were first fixed with methanol for about 30 seconds by taking precautions not to pour on the thick film. A fresh 10% Giemsa solution in tap water were prepared and slides were immersed into staining jar for 10 minutes. The stained blood films were rinsed by tap water and put in upright position to dry.

3.3.3. Handling and Transportation of the Samples

Two stained thick and thin blood film slides from each patient were put into slide boxes. The air-dried WFP spotted blood samples were transferred to a re-sealable plastic bag carrying the same label as the papers. The samples were transported to at ambient temperature (Paul *et al*, 1998; Wooden and Kyes, 1993) to the AHRI/ALERT laboratory, Addis Ababa, where all laboratory work was done.

3.3.4. Microscopy

Giemsa stained thin and thick films were observed under 100x objective light microscope by oil immersion. A sample was considered negative only after 200 microscopic fields were read without parasites. The parasite density was assessed by counting the number of parasites per 200 leukocytes in an oil immersed thick film. Then, the counted value was changed into per µl value by the following formula: (Farnert *et al*, 1997)

Number of parasites / Number of WBCs X 8000 $\Rightarrow$ Number of parasites X 40 µl $\Rightarrow$ Number of parasites per µl

3.3.5. DNA extraction from blood spotted filter paper

The blood-spotted filter paper was cut to appropriate size (around $3mm^2$) using a razor blade. Dried, blood-blotted filter papers were put in 1 ml of PBS (pH 7.4) and 50µl of 10% saponin in 1.5 ml microfuge tubes. The microfuge tubes were inverted several times, and stored overnight at 4^OC. After incubation, microfuge tubes were spun for 5 seconds, and the now reddish PBS/ saponin solution were aspirated from the tubes. Then, 1ml of PBS/ tube (no saponin) was added, inverted several times, and incubated at 4^oC for 1-2 hours.

After spinning the microfuge tubes for 5 seconds, as much fluid as possible was aspirated from the tubes. Then, 100µl sterile water was added to each tube. In samples with very small or very thin blood blot, and/ or with low parasitaemia (e.g., < 1,000/µl), 50µl of sterile water was added instead of 100µl. Then, vortexed Chelex stock suspension was transferred to a 1.5ml microfuge tube, and 50µl was dispensed to each sample using a tip with its tapered end cliped of by vortexing or inverting the tube after every three or four transfers, not to transfer just the water, with all the Chelex settled to the bottom of the dispensing tube. The parasite DNA was extracted by incubating tubes for 10 minutes in a 95 ^0C water bath by vigorously

vortexing each sample every two minutes or so throughout the incubation to increase DNA yield. Each tube was uncapped after every two minutes to allow for release of air-pressure.

After incubation, the tubes were microfuged for 5 minutes at 14,000 rpm. Meanwhile, two sets of 1.5 microfuge tubes were labeled for transfer, the second set for final storage of the extracted DNA samples. From the spun tubes, as much solution as possible was transferred to the first set of microfuge tubes with 200µl tip, not worrying if chelex is carried over as well. The tubes were spun for 10 minutes, then, white-to-yellowish supernatant was transferred to the final set of labeled tubes by avoiding the pelleted chelex. The tubes were stored at –20^{O}C until used for PCR analysis.

3.3.6. Genotyping of *P. falciparum* by Family specific nested PCR

P. falciparum DNA was amplified by Nested-PCR using primers for MSP-2 genes (Snounou *et al*, 1999) (Table 1).

Table 1: Primer sequences used for amplification of MSP-2 gene in *P. falciparum*.

Primer	Sequence	Notes
MSP-2 CF	5’ ATGAAGGTAATTAAAACATTGTCTATTATA3’	Conserved
MSP-2 CR	5'-CTTTGTTACCATCGGTACATTCTT 3’	Conserved
MSP-2 FCF	5'-GCAAATGAAGGTTCTAATACTAATAG 3’	FC27 family-specific
MSP-2 FCR	5'-GCTTTGGGTCCTTCTTCAGTTGATTC	FC27 family-specific
MSP-2 ICF	5'AGAAGTATGGCAGAAAGTAAGCCTCCTACT	3D7 family-specific
MSP-2 ICR	5'-GATTGTAATTCGGGGGATTCAGTTTGTTCG	3D7 family-specific

.3.6.1. Polymerase Chain Reaction

In order to prevent cross-contamination, distinct work areas were used for DNA template and mix preparation and DNA amplification. A negative control without DNA template was included in each set of PCR amplifications. Moreover, one uninfected blood sample (from the investigator) was included for every fifteen samples processed. Around ten percent of positive-PCR samples were re-tested to confirm the presence of plasmodial DNA at AHRI and Karolinska Institute, Sweden by different individual. To each nested PCR reaction one positive and one negative control tests were run. For MSP-2 gene amplification laboratory strains F32, NF54, and K1 were used as a positive control. For PCR negative samples the PCR run was repeated. Amplification was performed using standard PCR buffer.

In general, a 20µl reaction mix contained the following components: 10µl HotStart **Taq** ® **Master** Mix (**Qiagen** Inc., CA, USA), 250nM of each primer and 6µl purified DNA was used in primary reaction and 1µl of PCR product was used for 5 separate nested reactions.
The PCR conditions were as follows (Snounou *et al*, 1999):

a) . Primary Reaction

Stage 1: Initial denaturation step of 5 min at 95ºC (1 cycle)

Stage 2: (25 cycles)

Step 1: Denaturation for 1 min 94ºC

Step 2: Annealing for 2 min at 58ºC

Step 3: Extension for 2 min at 72ºC

Stage 3: Final elongation step for 5 min at 72ºC (1 cycle)

b). Nested reaction:

Stage 1: Initial denaturation step of 5 min at 95ºC (1 cycle)

Stage 2: (30 cycles)

Step 1: Denaturation for 1 min at 94ºC

Step 2: Annealing for 2 min at 61ºC

Step 3: Extension for 2 min at 72ºC

Stage 3: Final elongation step for 5 min at 72ºC (1 cycle)

3.3.6.2. Electrophoresis of PCR Products

Two percent agarose gel in TAE buffer (Glacial Acetic Acid, Tris-base, and EDTA) was prepared in 50ml volume by adding 1µl of 10mg/ml of Ethidium Bromide. After addition of 2µl of 6x gel loading solution to the amplified product, 10µl was analyzed by agarose gel electrophoresis in TAE buffer for an hour. Then, PCR products on a gel were visualized by UV Transluminator and photographed for documentation (Bioimaging Systems ® , UVP Laboratories).

3.3.7. Genotyping *P. vivax* by PCR-RFLP

P. vivax DNA was also amplified with the following primers for MSP3α gene (Bruce *et al*, 1999):

Name	Sequence
VP1 (fwd)	5' CAG CAG ACA CCA TTT AAG G 3'
VP2 (rev)	5' CCG TTT GTT GAT TAG TTG C 3'
VN1 (fwd)	5' GAC CAG TGT GAT ACC ATT AAC C 3'
VN2 (rev)	5' ATA CTG GTT CTT CGT CTT CAG G 3'

3.3.7.1. Polymerase Chain Reaction

Nested PCR amplification of the genes was carried out in reaction volumes of 20µl using 10µl HotStart **Taq**® **Master** Mix (**Qiagen** Inc., CA, USA), 1-2µl of DNA extracts in the primary round and 1µl of the primary PCR product in the nested round and 0.25µM of each primer (Bruce *et al*, 1999).

a). Primary Reaction

Stage 1: Initial denaturation step of 5 min at 94ºC (1 cycle)

Stage 2: (35 cycles)

Step 1: Denaturation for 30 seconds at 94ºC

Step 2: Annealing for 30 seconds at 56ºC

Step 3: Extension for 2.5 minutes at 68ºC

Stage 3: Final elongation step for 2.5 min at 68ºC (1 cycle)

b). Nested reaction:

Stage 1: Initial denaturation step of 5 min at 94ºC (1 cycle)

Stage 2: (30 cycles)

Step 1: Denaturation for 30 seconds at 94ºC

Step 2: Annealing for 30 seconds at 57ºC

Step 3: Extension for 2.5 min at 68ºC

Stage 3: Final elongation step for 2.5 min at 68ºC (1 cycle)

3.3.7.2. Electrophoresis of PCR Products

One percent agarose gel in TAE buffer (Glacial Acetic Acid, Tris-base, and EDTA) was prepared in 50ml volume by adding 1µl of 10mg/ml of Ethidium Bromide. After addition of 2µl of 6x gel loading solution to the amplified product, 2µl of it was analyzed by agarose gel electrophoresis in TAE buffer for an hour. Then, PCR products on a gel were visualized by UV Transluminator and photographed for documentation (Bioimaging Systems ® , UVP Laboratories).

3.3.7.3. Restriction Digest

Six microliter *P. vivax* positive PCR product was digested with the restriction enzymes *Hha I* in 20µl reaction volumes (5 units of enzyme per reaction) in buffer supplied with enzymes at 37ºC for 4 to 5 hours. The restriction fragments were separated on 1.8% agarose gel. For determination of fragment lengths a 100 bp DNA ladder was used. Individual clones were identified by allele-specific patterns (Farnert *et al*, 2001).

3.5. Ethical considerations

The project was discussed with concerned bodies in the study sites and their agreement obtained. Support letters were then received and submitted to the ethics committees. The project obtained ethical approval from the ethical committees of AHRI/ALERT School of Graduate Studies, Addis Ababa University and National Ethical Clearance Committee of the Ethiopian Science and Technology Commission. The project was not started before ethical clearance was obtained from these committees. Copies of ethical clearance paper were submitted to the managements of health care facilities selected for the study and their permission was obtained before starting the whole procedure of blood specimen collection. The whole procedure at the study sites was coordinated by the local managements and carried out during the working hours of the institutions.

Blood samples were collected in health care facilities where the patients came to seek health care. So, blood sample collection did not interfere with the normal daily routines of the patients since they were present because of the illness itself. When patients come to health care facilities for malaria symptoms, they know that a blood specimen will be taken from them for diagnosis. In this study, no invasive procedure other than the finger pricking, which is used for routine diagnosis, was used. An additional requirement different from the routine diagnosis is that; more volume of blood was needed for two additional blood film slides and spot on Whatman filter paper that did not exceed 500µl. Before blood sample collection, the

patients were informed clearly about the objectives of the study and the procedures they should follow. Their agreement was confirmed by putting signature on the consent form written in Amharic and witnessed by independent people. For those who did not speak Amharic, oral translations were provided.

The patients were examined by experienced health officers working at the site for diagnosis. Case record forms were kept confidential. Finger pricking procedure was performed by experienced laboratory technicians to avoid unnecessary pain and bleeding. For finger pricking, single disposable sterile lancet was used per person to avoid possible transmission of infection with blood borne pathogens such as HIV and Hepatitis B Virus. The investigator made sure that the patients were well informed about their laboratory results and treated properly. Patient received their results from the slides checked by the routine diagnostic tests in the institution. There was no delay in patient care because of the study. The participants were supplied with anti-malarial drugs according to the guidelines of Ethiopian Ministry of Health free of charge.

3.6. Data Management, Interpretation and Analysis

Socio-demographic data from study subjects, data from case record forms and laboratory results were entered into a database using Microsoft Excel and by Data Management Center at AHRI. EpiInfo 3.3.2 (WHO/CDC, 2005) statistical package was used for cleaning and analysis of the data.

An MSP-2 and MSP-3α positive reaction was interpreted as an indication for the presence of *P. falciparum* and *P. vivax* parasites in the sample, respectively. Assignment of a PCR fragment to a specific allelic family was based on the result of secondary PCR using family specific primers for *P. falciparum*. The minimum number of genotypes was enumerated as total number of bands within the marker. Since the targeted regions are unlinked, the number of bands in one region is independent of the number of bands in other regions.

Multiplicity of infection (number of bands per infected person) was calculated as the average number of distinct parasites per PCR positive sample. The distribution of various allelic families was estimated by dividing the total number of fragments assigned to one family detected in the typing reactions by the total number of fragments for the locus considered. Within each allelic family, alleles were differentiated by size differences following electrophoresis of PCR amplified products. The molecular size of the bands was determined by lab works 4.0 software (Bioimaging Systems®, UVP Laboratories) and the size of the bands was rounded off to 20 base pair differences to minimize size variation due to band intensity.

Comparison of multiplicity of infection with age, parasite density, and severe disease was done by X^2 tests. (Magesha *et al*, 2001; Magesha *et al*, 2002, Farnert, *et al*, 2001, Cui *et al*, 2003; Basco, *et al*, 2004).

4. Results

4.1. Study Population

Blood samples were obtained from a total of 301 patients. Two hundred and ten miroscopically confirmed malaria cases, as diagnosed by the local laboratory technician and/or investigator at AHRI, from patients attending health institutions with clinical symptoms of malaria were recruited in the study. In areas where there was no microscope (Derra excluding Gundomesqual Health Center), case record forms were filled and blood samples were taken from clinically diagnosed malaria patients. Then, the blood film slides were examined by the investigator and microscopically negative patients were excluded from the study.

The distribution of study participants was 61 patients from Arbaminch, 52 from Burie, 40 from Derra and 57 from Zeway were the study participants. The age of the participants ranged from 6 months to 60 years with a mean age of 18.1± 14.1 years. The mean age of patients was 15.1 ± 14.4, 18.2 ± 12.1, 25.9 ± 13 .7, and 15.5 ± 14.0 in Arbaminch, Burie, Derra, and Zeway, respectively. Seventeen point nine percent (n=37) of the study population were under 5 years of age, with 110 males and 99 females participating in the study. The most frequent ethnic groups were Amhara and Oromo, accounting 47.5% and 26.1% of the total study population, respectively (Table 2).

Table 2: Demographic information about the study participants from Arbaminch, Burie, Derra, and Zeway, in 2004/2005

Socio-demography	Arbaminch	Burie	Derra	Zeway	Total n (%)
Age (in years)	16	9	1	13	37 (17.9)
< 5	12	5	4	13	34 (16.4)
5 – 9	15	14	11	11	51 (24.6)
10 – 19	8	13	10	8	39 (18.8)
20 – 29	2	9	6	8	25 (12.1)
30 – 39	8	2	8	3	21 (10.1)
> 40	61	52	39	55	207* (100)
Total					
Sex	33	30	21	26	110 (52.7)
Male	28	22	19	30	99 (47.3)
Female	61	52	40	56	209* (100)
Total					

4.2. Clinical Parameters of Malaria in the Study Population

Almost 45% of microscopically confirmed (at local sites) malaria patients had an axial temperature of 37.5^{0}C and below. The mean temperature measurement value was 37.7^{0}C $\pm$ 1.12. Although 86% of the malaria positive complained having fever as symptom during one week period before the time of consultation. Again 90.7 % of the patients experienced common symptoms of malaria (myalgia, chills, loss of appetite, head ache) while the rest 9.3 % (n =19) did not experience any of the symptoms.

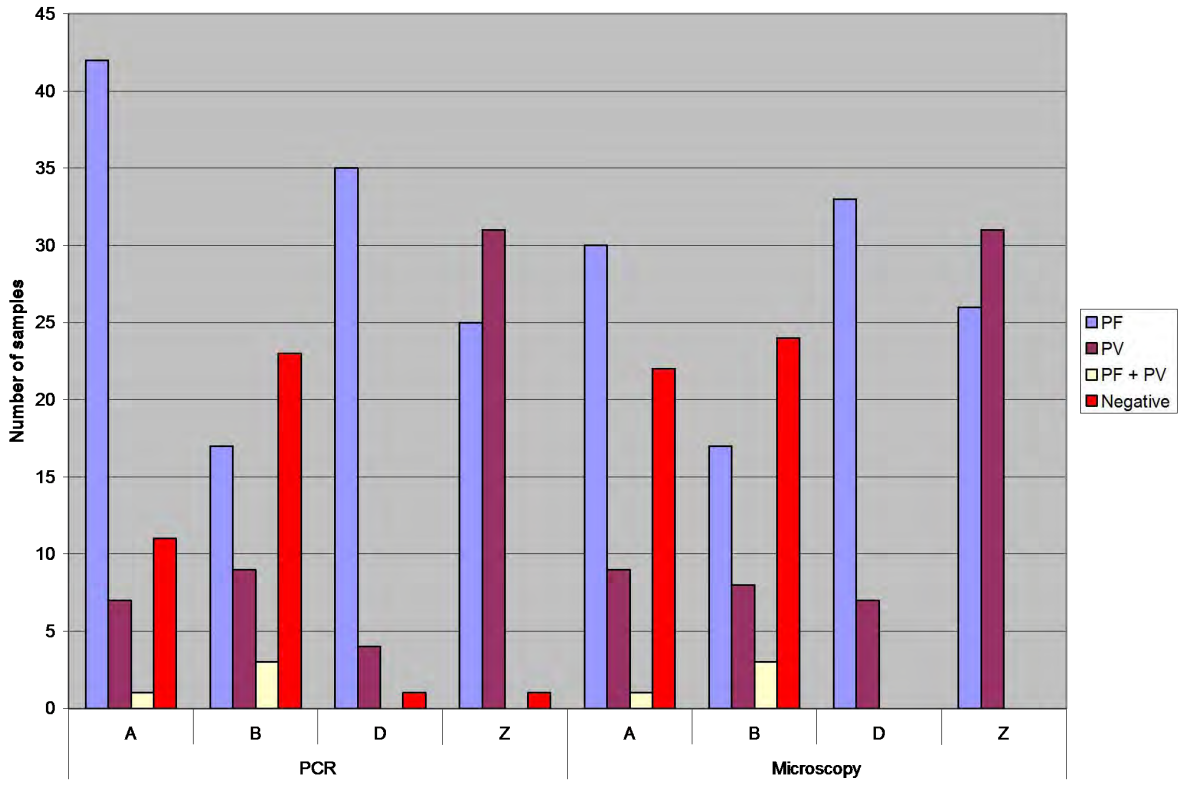


Figure 6: Malaria parasite species prevalence by microscopy and PCR in the four study sites, in 2004/2005.

A= Arbaminch, B=Buire, D = Derra, Z = Zeway, PF = *P. falciparum*, PV = *P. vivax*, PF + PV = mixed *P. falciparum* and *P. vivax* infection.

The majority (66.3%) of the patients had more than one malaria episodes, before the current illness (The number of episodes was not recorded because the exact number of episodes might not be recalled). Among the study participants, 37.4% of the patients had never been treated with antimalarial drugs before. Of 127 patients who were examined for spleen enlargement, enlarged spleen was detected in only nine (7.1%) patients. The proportion of severe malaria cases was 3% (n = 6) as defined by examiners at the health institutions.

4.3. Parasite Prevalence

All reported microscopy positive samples (according to the diagnosis of local laboratory technicians and the investigator) for either of the two dominant species *P. falciparum* and *P. vivax*, were genotyped by PCR. Of cases reported positive by local laboratory technicians,17.5% and 21% were negative by PCR and microscopy at AHRI, respectively. Twelve samples which were negative by microscopy were positive by PCR and 3 samples that were reported positive by microscopy were negative by PCR despite repeated attempts starting from re-extraction of DNA. Almost all samples except one, which was microscopy positive and PCR positive from Zeway were positive by both microscopy and PCR. Almost half of the samples from Burie and one sixth from Arbaminch were false positive as compared to PCR results.

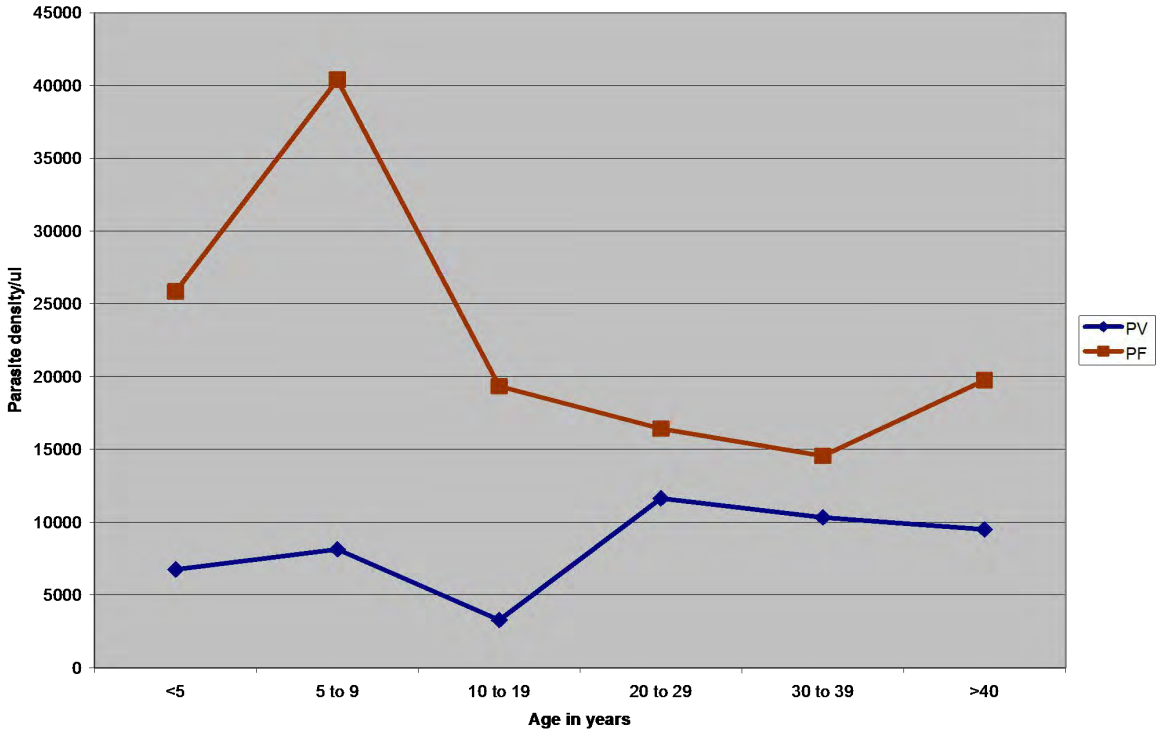


Figure 7: Mean parasite density of *P. falciparum* and *P. vivax* in different age groups.

Species prevalence was 64.5% (107/166) *P. falciparum*, 33.1% (55/166) *P. vivax* and 2.4% (4/166) mixed *P. falciparum* and *P. vivax* according to microscopic diagnosis at AHRI and 68.2% (118/173) *P. falciparum* and 29.5 % (51/173) *P. vivax* and 2.3% (4/173) mixed *P. falciparum* and *P. vivax* by PCR (Fig. 6). No *P. malariae* and *P. ovale* species were detected in any of the samples.

Out of 208 samples, 161 were positive both by microscopy and PCR, 12 were positive by PCR assay alone and three samples were positive by microscopy alone. As compared to microscopic diagnosis, the sensitivity and specificity of PCR was 98.1% and 72.7% (Table 5), respectively. The sensitivity and specificity of microscopy was 93.1% and 91.4%, respectively when PCR method was used as a reference.

The mean parasite density of both parasite species was 18,191.4 ± 40,227.2 parasites/µl of blood (range: 40 to 400,000 parasites/µl). Species-specific parasite density of 23,168 ± 48,621.1 parasites/µl and 7,721.1 ± 8,659.6 parasites/ µl for *P. falciparum* and *P. vivax,* respectively (Fig. 7). The mean parasite density for mixed *P. falciparum* and *P. vivax* infections was 21,370 ± 34,310 parasites/µl. The highest (28,951.8 ± 76,391.9 parasites/µl)

and the lowest (13,015.4 ± 20,454.2 parasites/μl) mean parasite density was recorded in patients of 5 to 9 and 30 to 39 years of age, respectively.

Table 3: Sensitivity and Specificity of PCR and Microscopy.

A
Microscopy

PCR	**Positive**	**Negative**	**Total**
Positive	161	12	173
Negative	3	32	35
Total	164	44	208

B
PCR

Microscopy	**Positive**	**Negative**	**Total**
Positive	161	3	164
Negative	12	32	44
Total	173	35	208

A: Sensitivity 161/164 = 98.1 %, specificity 32/44 = 72.7%, of PCR by taking microscopy diagnosis as a gold standard

B: Sensitivity 161/173 = 93.1 % and specificity 32/35 = 91.4 % of microscopy by taking PCR as a reference.

4.4. Parasite Genotyping

A total of 173 samples were genotyped in this study with amplification success of 97.7%. Of these samples, 122 were analyzed for MSP-2 polymorphism in *P. falciparum* and 55 for MSP 3α gene polymorphism in *P. vivax.* This figure includes mixed *P. falciparum* and *P. vivax* infections in polymorphism study of both genes.

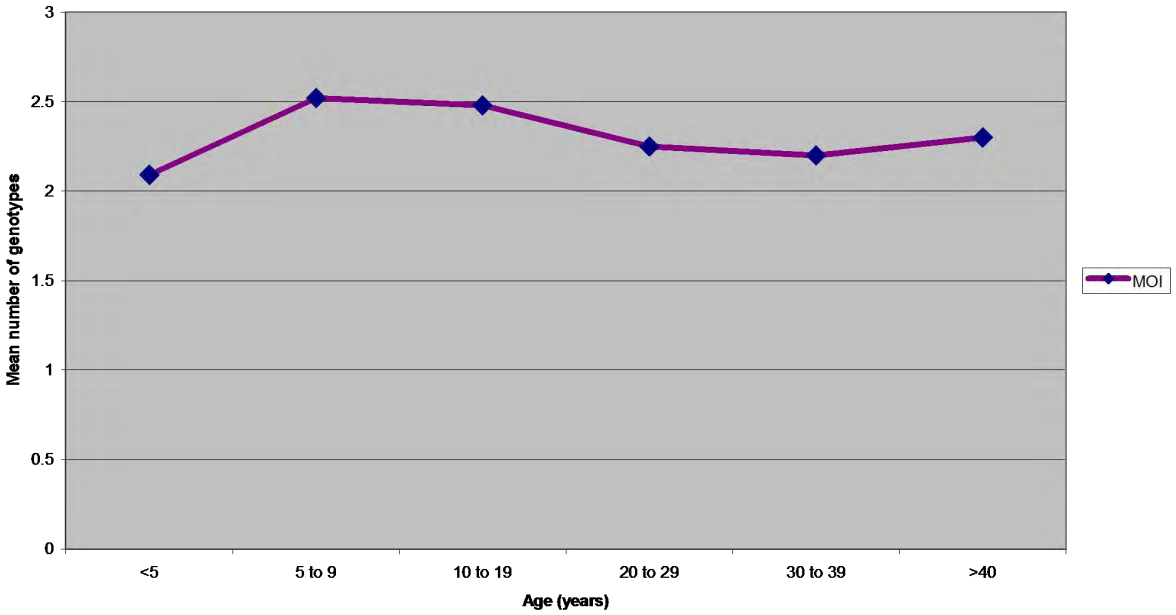


Figure 8: Age distribution of multiplicity of infection using MSP-2 gene of *P. falciparum.*

4.4.1 Genotyping *P. falciparum*

4.4.1.1. Prevalence of Multiple Infections

Since blood forms of malaria parasite are haploid, the presence of more than one allele (band) in the gene indicates the occurrence of infection with more than one genotype.

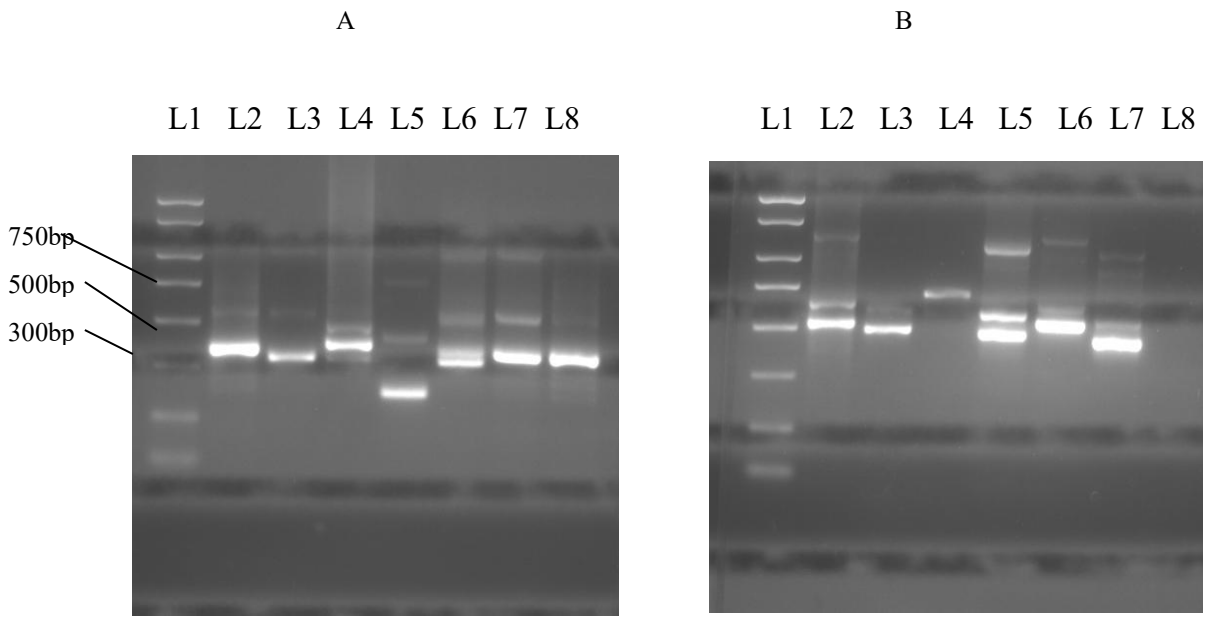


Figure 9: A gel picture of *P. falciparum* MSP 2 gene Nested PCR amplified product.

Lane 1: DNA ladder, Lane 2-7 field samples. Lane 8: Positive control K1 A: FC27 allelic family. B: IC-1 allelic family

Of 122 isolates genotyped, n=95 (78.5%) consisted of multiple infections with at least two different clones. The large majority of the patients (n=53) had double infections with either of FC27 and/or IC-1 allelic families, 27 (22.3 %) isolates had single or multiple infections only with FC27-type allelic family, 49 (40.5%) had single or multiple infections belonging only to IC-1 type allelic family. Multiple infections within parasite population is due to infection by both allelic families. Out of four mixed *P. falciparum* and *P. vivax* infections, three isolates had genotypes of only IC-1 allelic family and the remaining one isolate had single infection by FC27 allelic family. Of 262 fragments, 134 (51.1 %) and 128 (48.9%) belonged to FC27 and IC-1 allelic family, respectively.

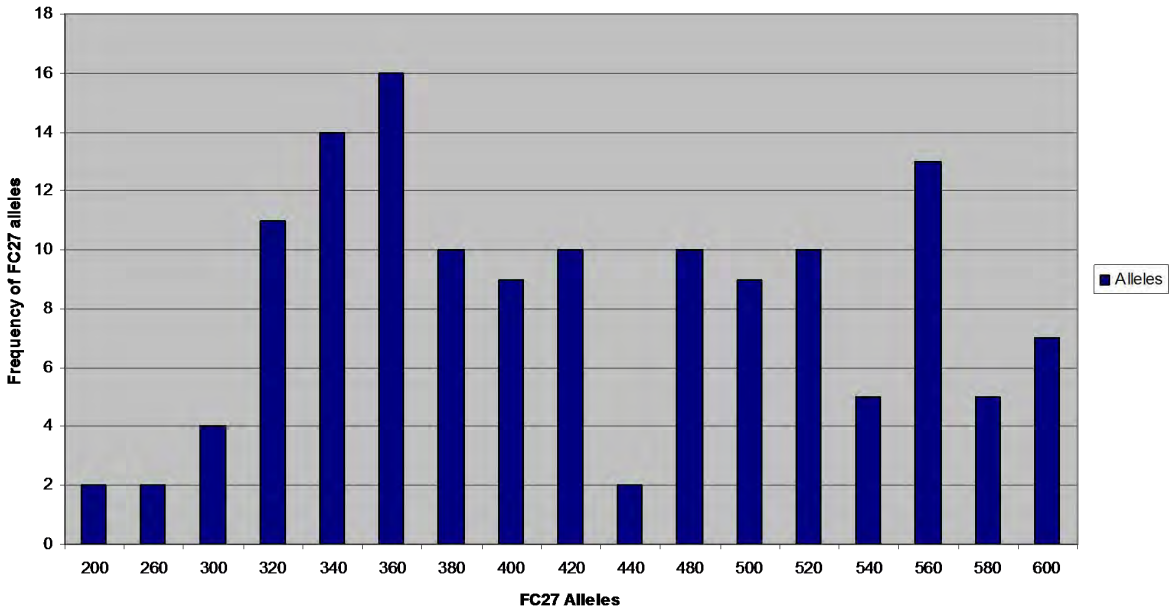


Figure 10: Allelic Frequency of FC27 allelic family of MSP-2 gene in *P. falciparum*

4.4.1.2. Allelic Diversity in MSP 2 Gene

There were 37 different alleles of MSP 2, comprising 17 (45.9) FC27 type alleles (figure 10) and 20 (54.1%) IC-1 type alleles (figure 11), as determined by the number of different size fragments belonging to each allelic family. The two most frequent alleles of FC27 allelic family were 360 bp, 340 bp and 560 bp alleles accounting for 11%, 9.6% and 9.6% allelic frequency, respectively. In IC-1 type allelic family, 460 bp and 480 bp alleles were the most frequent alleles comprising 11.8% and 9.7% allelic frequency, respectively (figure 10 and 11). None of the alleles of the two allelic families occurred only once.

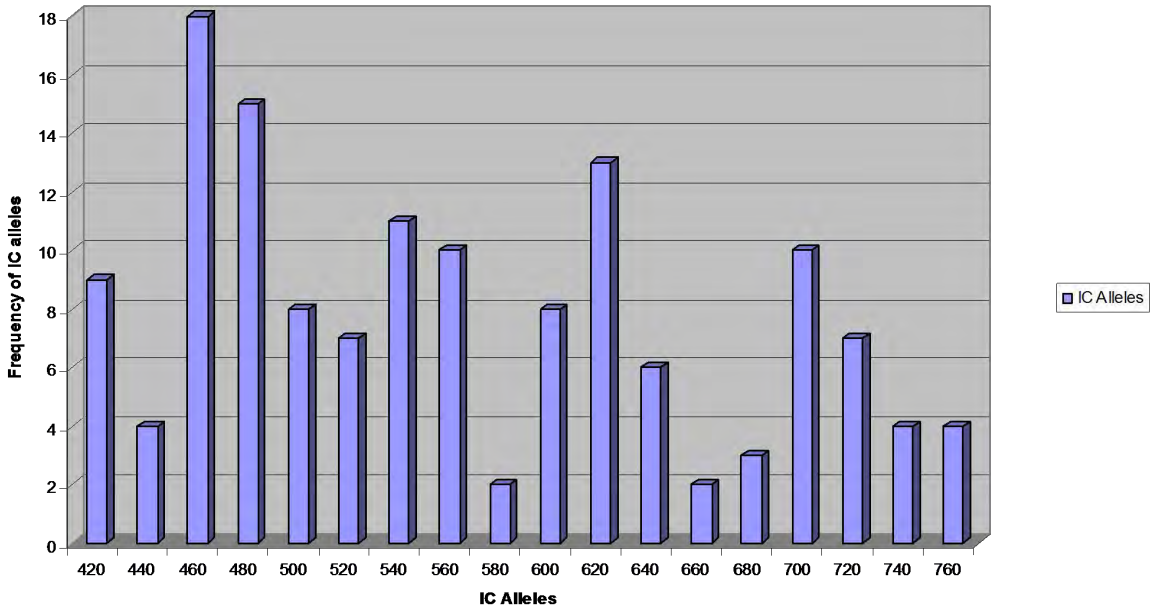


Figure 11: Allelic frequency of IC-1 allelic family of MSP-2 gene in *P. falciparum*

4.4.1.3. Multiplicity of Infection

The mean number of genotypes in all age groups of patients in the four study sites was 2.34 ± 1.10 (range 1 - 6) per individual. The mean value was higher in patients of 5 to 9 age group and in patients from Derra with 2.52 ± 1.28 and 2.51 ± 1.01, respectively (Table 4). Neither age *(P value* = 0.8693) nor parasite density (*P value* = 0.1913) had an association with MOI.

Table 4: Age group distribution of mean number of genotypes in the four study sites.

Age Group (years)	Mean number of genotypes				
	Arbaminch	**Burie**	**Derra**	**Zeway**	**Total**
<5	2.8	2.33	2.0	2.0	**2.09**
5 – 9	3.0	2.33	2.25	2.0	**2.52**
10 – 19	2.25	2.33	2.7	2.75	**2.48**
20 – 29	2.0	2.0	2.57	2.2	**2.25**
30 – 39	1.5	2.5	3.0	1.66	**2.2**
≥ 40	2.33	-	2.43	2.33	**2.3**
All	**2.32**	**2.26**	**2.51**	**2.16**	**2.34**

P value = 0.8693

4.4.1.4. Association between multiclonal *P. falciparum* infection and Clinical parameters

Multiple infections were associated with seven different clinical parameters in the univariate analysis. The parameters were: age, sex, residence, fever, parasitemia, clinical diagnosis, and history of repeated malarial attack. Only sex and geographical location had statistically significant influence on multiplicity of infection. Other important variables like age, clinical diagnosis, fever, and parasitemia had no influence on multiplicty of infection (Table 5). In severe malaria cases, all infections were multiclonal but X^2 (*P value* = 0.1948) test result showed non significant difference between severe and non severe malaria cases

Table 5: Association of multiple infections and clinical parameters.

Parameters	No	Proportion with > 1 genotype	X^2 P
Residence			
Arbaminch	43	65.0	
Burie	19	78.9	0.0410
Derra	34	91.2	
Zeway	25	84.0	
Age			
< 5	21	71.4	
5 – 9	21	85.7	
10 – 19	29	79.3	0.870
20 – 29	20	73.7	
30 – 39	15	80.0	
> 40	16	81.3	
Sex			
Male	68	70.6	0.016
Female	53	88.7	
Fever (T^O >37.5^{O}C)			
Yes	65	83.7	0.1928
No	54	73.5	
Clinical diagnosis			
Complicated	6	100.0	0.1948
Uncomplicated	115	77.9	
Parasitemia			
40 – 999	31	61.3	
1000 – 100,000	74	87.8	0.1032
> 100,000	5	60.0	
Repeated malaria attack (>2)			
Yes	79	83.5	0.1011
No	41	70.7	

4.4.2. Genotyping *P. vivax*

Almost all (98.2%) microscopically confirmed *P.vivax* positive samples were successfully amplified by PCR using MSP-3α gene as a marker for genotyping. Based on the size of PCR products there were 3 major types: ~ 1.9Kb size, 1.5Kb size, and 1.1Kb. 1.9 Kb type was the most dominant allele accounting for 78.2% of all types. The other two types, 1.5 Kb and 1.1 Kb, accounted for 7.3% and 14.5%, respectively. All four mixed *P. falciparum* and *P. vivax* infection isolates had only the 1.9Kb type.

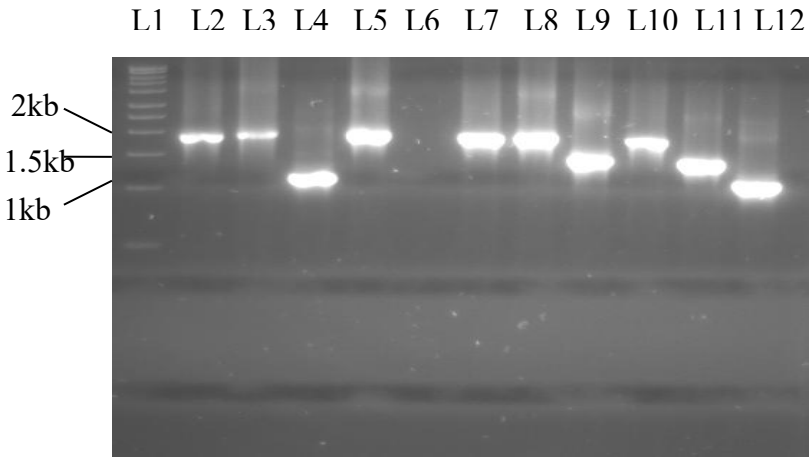


Figure 12: Undigested PCR products of MSP 3 gene of *P. vivax*

Lane 1: DNA ladder, Lane 2- positive control. Lane 3-12 field samples

The PCR-RFLP analysis of msp3 further demonstrated that *P. vivax* isolates in Ethiopia were highly diverse. By using restriction pattern of *Hha I* digestion, it was possible to identify 14 alleles in 55 patients: 12 alleles from 1.9Kb type, only 1 allele from each of 1.5 and 1.1Kb types. In this genotyping method, 4/55 (7.2 %) of patient isolates were found to have

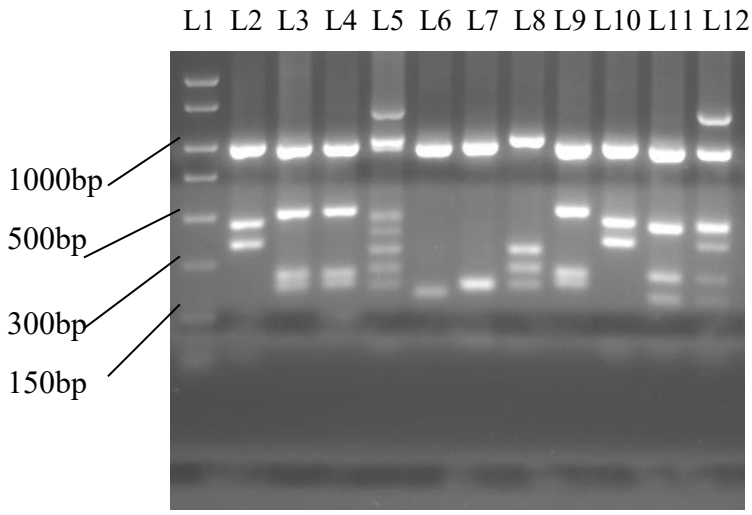


Figure 13: PCR-RFLP products of *Hha I* digestion of MSP-3α gene of *P. vivax*

Lane 1: DNA ladder, Lane 2-12 field samples

multiple infections with different genotypes of *P. vivax.* The type of alleles in multiple infection samples could not be differentiated. The PCR- RFLP patterns of all samples showed a band of approximately 1.0 Kb. Although this band is also slightly polymorphic, it was not included for distinguishing different msp3 alleles. Smaller bands ranging from 100 to 600 bp were used for RFLP analysis. By comparison, it was observed that at least five alleles were common from previous studies in Asia.

5. Discussion

The age distribution is a key indicator of endemicity and the acquisition of natural immunity to malaria parasites. The mean age of the patients was 18.1 $\pm$ 14.1 years with a median value of 16 with range of 6 months to 60 years. The seventy five-percentile value was 26 years. Almost half of the study population was 15 years and below. Such a figure is a characteristic of hypoendemic and seasonal malaria transmission areas. Furthermore, all age groups were infected to the same extent even though more cases were among the younger populations. Similar findings were reported from in hypoendemic and seasonal transmission areas from Sudan (Arnot, 1998).

The finding that malaria patients from Derra were older and those from Arbamich were younger than all other sites is an indication of the variation in the intensity of malaria transmission in the study locations. However, health care seeking behavior in both communities should be taken into consideration. According to the reports of the respective Woreda Health Bureaus, malaria transmission in Derra is mostly seasonal and low. In Arbaminch, it is more or less all year round with some seasonal peaks. In perennial and high malaria transmission areas the chance of developing acquired immunity to the disease is higher at the later stage of life due to longer exposure. As a result, adults are less affected by the infection. But in low and seasonal malaria transmission areas all age groups are at risk of developing the disease because of reduced stimulation of the immune system to infection.

It was interesting that almost forty-five percent of patients had no detectable fever by axial temperature measurement although majority of patients had history of sudden on set of fever. This situation can be explained by the nature of fever in malaria and the usage of thermometer used. A period of subsequent high fever followed by chills is a common manifestation of malaria illness. At the time of physical examination the patients might be in low fever state so that fever could not be detected at that time. In addition, inadequate time duration used for measuring temperature and improper placement of the thermometer may have impact on temperature measurement. We have however tried to avoid such an error.

Although the measurement was possibly subject to observer variation, spleen enlargement rate was 7.1 % which is very low when compared to other areas in West Africa (Bereczky *et al*, 2005: unpublished). Splenomegally is used as a classical indicator of endemicty. Enlargement of spleen may be detected a few days after an acute attack of malaria non-immune or in semi-immune individuals. It then gradually decreases after recovery from disease (Bryceson *et al*, 1983). In areas of high transmission, persistent splenomegally is found in a high proportion in apparently healthy children. Such low enlarged spleen rate may be due to: either the study areas are hypoendemic (< 10%) for malaria (Zimmerman *et al*, 2004). If patients came early to health care service before spleen enlargement could be noticed, the rate of splenomegaly may also be lower.

Almost one fifth of the samples reported positive by laboratory technicians in respective study clinics and recruited into the study were negative by both microscopy and PCR at AHRI. In Zeway (Battu) Health Center where Giemsa stained blood film slides were read by a specially trained laboratory technician of the Malaria Control Department the results microscopy reading better agreed with both microscopy and PCR at AHRI. Therefore, such a high rate of false positive results in other study sites could be explained by lack of experience in reading blood film slides for detection of malaria parasites or deterioration of sample quality during transport to AHRI

Nested-PCR using DNA extracted from filter paper assay was very sensitive (98.2%) but its specificity (72.7%) was low when the microscopy result at AHRI in our hands was used as gold standard. In a study which compared PCR using DNA from different blood storage devices and microscopy, the sensitivity and the specificity of PCR using DNA from filter paper was 65% and 93%, respectively (Scopel *et al*, 2004), The low sensitivity of PCR may be related to false reporting of negative samples as positive. Moreover, because PCR is more sensitive than microscopy, microscopy may not be appropriate to measure false positivity (Roper *et al*, 1996, Zwetyenga *et al*, 1999; Färnert *et al*, 1999; Magesh *et al*, 2002). When we take PCR as a reference, the specificity (91.4%) and sensitivity (93.1%) of microscopic diagnosis was good. This finding shows that microscopy is a good tool for diagnosis of malaria if it is carefully observed.

Regarding species identification, PCR was good in distingushing between the two species as reported by other studies (Roper *et al*, 1996; Roshanravan *et al*, 2003). The application of PCR revealed the prevalence of *P. falciparum* in Derra (82.5%) to be very much higher than that was reported by Derra District Health Office (less than 10 %). There is only one solar microscope in the district at Gundomesquel Health Center which is expected to serve such a large population in the whole district. In addition, there is much geographical inaccessibility (bad roads and far distance). In the health center only 20 to 30 slides are read in a week on average. This shows how low the patient flow is. The report of the District Health Office relies mainly on Gundomesqual Health Center data and on rare epidemic surveys which may not be informative for the whole district and underestimate malaria prevalence. In this study, samples were obtained mostly from four rural health posts two of which were at a distance of approximately 30 to 50 kms from the health center. However, the annual seasonal variations should be considered here. The annual pool may have a different proportion compared to the short survey we did.

The proportional prevalence of *P. falciparum* and *P. vivax* in general was more or less similar with the average national figure of 60 to 70 % and 30 to 40 %, respectively (MOH, 2005; Wondatir *et al*, 1992). However, in Zeway the dominant species was *P. vivax* accounting for more than half of the total prevalence. In the rest of study areas *P. falciparum* was the dominant species. All samples from Zeway were collected during the dry malaria transmission season when *P. vivax* is known to be dominant. According to the report of Zeway Malaria Control Department, *P. falciparum* is more prevalent during wet malaria transmission seasons and during epidemics (Ato Hailu, personal communication, 2004).

The finding that *P. vivax* malaria patients had much less parasite load than *P. falciparum* malaria patients is related to the biology of the parasites. *P. falciparum* can multiply into enormous numbers without causing any symptom (Zimmerman *et al*, 2004). In addition, highest parasite density was detected in 5 to 9 and under 5 years old children in both parasite species. As it was shown a study in high malaria transmission area, high parasite density was observed in under five years of age children (Owusu-Agyei *et al*, 2002). In the adults low parasite density was observed. As age grows old in endemic areas, the chance of being

exposed to malaria infection increases. Therefore, adults become semi-immune to the infection and they can limit multiplication of parasites.

Many of the previous studies on field isolates of *P. falciparum* compared size variation of PCR products based on agarose gel electrophoresis and/or hybridization with probes to demonstrate MSP-2 diversity (Felgel *et al*, 1999; Owusu-Agyei *et al*, 2002) . These methods are laborious and give a rough estimation of fragment size. Unless further analysis is made by RFLP and/or sequencing, they do not allow detailed analysis of parasite population structure. In this study, parasite genetic structure was analyzed using one of the best available means for characterizing multiple parasite populations i.e. family specific amplification. The most informative marker for the genetic diversity study of *P. falciparum* has been shown to be MSP-2, the marker used in this study (Färnet *et al*, 1999; Owusu-Agyei *et al*, 2002; Zwetyenga *et al*, 1999 Ntoumi *et al*, 1995; Aubouy *et al*, 2003).

The mean number of genotypes per isolate was 2.34. This is relatively higher as compared to low and seasonal malaria transmission areas in the Sudan which was reported 1.5 and of Senegal reported as 2.0 (Babiker, 1998; Zwetyenga *et al*, 1999). The rate is lower for a holoendemic area in Tanzania which was 2.7 in symptomatic and 4.9 in asymptomatic children (Magesha *et al*, 2002). Studies revealed that in clinical malaria the number of genotypes was lower than asymptomatic individuals (Engelbrecht *et al*, 1995; Farnet *et al*, 1997).

The number of MSP-2 alleles identified in this study is 37 out of 122. This is comparable with that reported from the Sudan (Babiker, 1998) which was 13/29 in 1989, 12/39 in 1990, and 1/43 in 1991. Similar reports included 25/61 from Brazil (Tonon *et al*, 2004), 38/144 from Papua New Guinea (Felger *et al*, 1994), and 50/108 alleles from Tanzania (Felger *et al*, 1999). This figure strongly illustrates the extent of genetic diversity in Ethiopian isolates of *P. falciparum*. Several studies suggested that the number and distribution of alleles may differ between symptomatic and asymptomatic individuals (Felger *et al*, 1999; Färnert *et al*, 1999). This may be important in identifying alleles responsible for morbidity and/or mortality.

The number of clones of malaria parasites co-infecting a single host is a useful indicator of malaria transmission and immune status of the host. Increase in the level of transmission is generally associated with progressive increase in the average number of malaria parasite clones per isolate (Arnot, 1998). Furthermore conducted in different transmission areas had indicated that the number of genotypes within a single host is higher in high malaria transmission areas (Paul *et al*, 1998; Ntuomi *et al*, 1995; Magesha *et al*, 2002). The increasing burden of malaria in Ethiopia nowadays may be associated with an increase in transmission intensity as indicated by the findings of this study.

Multiplicity of infection in this study was significantly different in the four study areas, with the proportion of multiple infection and mean number of genotypes being high in Derra as compared to other study sites. Although geographical difference in complexity of infection has been documented from other localities (Creasy *et al*, 1990; Walliker 1985), it is hard to explain how it was high in Derra where malaria transmission is low, and low in Zeway where malaria transmission is believed to be more intense. In fact, information on EIR is lacking to estimate the current intensity of malaria transmission in the study areas. It is possible that the interaction between the two species may be responsible in influencing parasite complexity. In Derra where the prevalence of *P. vivax* was low, the mean number of *P. falciparum* genotypes was the highest while in Zeway where *P. vivax* was the dominant species, mean number of genotypes was the lowest. Although adequate information is lacking on the interaction of the two species at a community level, different studies had reported that *P. vivax* infections help to reduce the severity of *P. falciparum* malaria (Zimmerman *et al*, 2004).

The findings of this study showed that age and parasite density were not significantly associated with multiplicity of infection. Similarly, areas of low endemicity like Ndiop in Senegal (Zwetyenga *et al*, 1999), The Gambia (Conway *et al*, 1991) and Sudan (Babiker, 1998) reported little or no influence on infection complexity and allelic distribution of *P. falciparum* infections. However, in many studies in areas of high transmission, mulitiplicity of infection has been shown to be age dependent, with the highest value reached in young children (Ntoumi *et al*, 1995; Owusu-Agyei *et al*, 2002). Therefore, such an association may be a useful indicator of the level of transmission. Neverthless, in a study in a meso to

hyperendemic malaria transmission area in Gabon such an association was reported as lacking (Aubouy *et al*, 2003).

The parasite isolates in this study, as in most field samples, contained several clones of polymorphic MSP-2 gene that considerably varied in size and repeat copy number. In such case, nested PCR is considered to be the best method to study MSP-2 polymorphism. Nevertheless, the results should be interpreted cautiously because of possible variations in sensitivity when dealing with weak bands and in estimating band size. Technical limitations such as use of different types of reagents, different amount and quality of DNA and unmanageable changes in temperature or moisture content, may lead to different results in different laboratories (Farnert *et al*, 2001: Aubony *et al*, 2003).

Almost all studies conducted on polymorphism of *P. vivax* were outside Africa where, in most cases, *P. vivax* infections are more prevalent than *P. falciparum* infections. However, the finding of this study revealed that *P. vivax* isolates in Ethiopia were diverse as what was documented from other countries (Bruce *et al*, 1999; Cui *et al*, 2003). The prevalence of three major MSP-3α types (1.9 kb, 1.5 kb and 1.1kb: 78.2%,7.3%, and 14.5%) was similar with what was reported from Thailand (70.5%, 6.7%, and 22.8%). Furthermore, the 14 MSP-3α alleles identified in this study are comparable to what was reported from Thailand (13) and Papua New Guinea (11).

This genotyping method used in this study identified only 7.2 % of vivax malaria patients to have multiple infections by different *P. vivax* genotypes. This figure is very low as compared to reports from Thailand and Papua New Guinea, which reported 19.3% and 22.7 % multiple infections, respectively, using the MSP-3α gene as a marker (Bruce *et al*, 1999; Cui *et al*, 2003). More alleles and multiple infections were detected by using more than one restriction enzyme in RFLP analysis and more than one polymorphic gene marker (Bruce *et al*, 1999; Severini *et al*, 2000). If we used more than one restriction enzyme, we could even identify more number of alleles.

From this observation, we can see that size difference of PCR products alone may not tell us the exact situation of *P. vivax* population structure. Inclusion of RFLP analysis of PCR

products increases the number of distinguishable alleles. Although it was easy to identify multiple infection by RFLP analysis of PCR products, it was hard to assign the multiple isolates to specific allele groups, as it was done for *P. falciparum* MSP-2 genotyping.

6. Conclusions

Blood sample storage on filter paper is very simple and useful for DNA isolation which could be used in PCR for genotyping studies on Plasmodium species.

PCR method identifies malaria parasite species better than light microscopy, which is liable to subjective judgment and needs ample experience.

Genotyping of MSP-2 gene of *P. falciparum* by nested-PCR has shown that Ethiopian isolates of *P. falciparum* are as highly diverse as those reported in other African countries where there is intense malaria transmission.

Neither host age nor parasite density of *P. falciparum* had an association with multiplicity of infection as reported from other hypo to mesoendemic malaria transmission areas where individuals have less acquired immunity to *P. falciparum* infection

Although it is difficult to conclude with such a small number of samples, PCR-RFLP analysis of MSP-3α gene in *P. vivax* showed that Ethiopian isolates of *P. vivax* are highly diverse with similar distribution of types with distant countries.

The existence of identical allelic types in Ethiopian isolates of *P. vivax* with allelic types in Asian countries may indicate genetic relatedness of *P. vivax* isolates in different countries.

7. Recommendations

Genetic diversity of both *P. falciparum* and *P. vivax* should be further investigated to better understand the complex interplay between multiplicity of infection and numerous host factors, in different epidemiological settings.

As the degree of exposure to infection may vary, even in one small community, depending on the preventive measures the individuals take and susceptibility, age may not be a good indicator of protection. Rather, studying degree of acquired immune response in relation to multiplicity of infection may provide a better measure of susceptibility.

To make comparisons between severe and non-severe malaria cases, case-control studies consisting of comparable number of severe and non-severe malaria cases may be the appropriate study design than the cross-sectional approach used in the present study to better understand the role of multiplicity of infection on pathogenesis of malaria.

Although it is difficult to routinely diagnose patients by PCR, as it requires equipped laboratory, and is time consuming and expensive, it is a necessary method for targeted epidemiological surveys as microscopy underestimates the burden of disease.

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Appendix A: Case Record Form

Study Clinic: _____ Date: _____

Identification No _____

A: Identification

Name: _____ Ethnicity: _____

Age(Yrs): _____ Religion: _____

Sex: male female

Residence: _____

B: Vital Signs

Temperature (°C): _____ Respiratory Rate (bpm): _____

Pulse Rate (bpm): _____ Weight (kg): _____

Blood Pressure (mmHg): _____

C: Patient Examination (Put mark in the right boxes)

	Yes	No
Sudden onset of fever		
Other symptoms of malaria		
History of antimalarial treatment		
History of repeated malaria attack		

Spleen not enlarged enlarged

Clinical Diagnosis Uncomplicated malaria Complicated malaria

Other Remark _____

D. Laboratory Form

	P. falciparum	*P.vivax*	Others	Negative
Microscopy				
HI*	☐	☐	☐	☐
AHRI*	☐	☐	☐	☐
PCR	☐	☐	☐	☐

Parasite density /µl _____

Genotyping of *P. falciparum*

MSP-2 FC27a _____ IC-1a _____

FC27b _____ IC-1b _____

FC27c _____ IC-1c _____

FC27d _____ IC-1d _____

Total Number of bands _____

Msp1 RO33a_____ MAD20a_____ K1a_____

RO33b_____ MAD20b_____ K1b_____

RO33c_____ MAD20c_____ K1c_____

RO33d_____ MAD20d_____ K1d_____

Total Number of bands _____

Genotyping *P. vivax*

Msp3α

Mol. Wt. _____ Type_____

*HI: Health Institution

AHRI: Armauer Hansen Research Institute

Appendix B

Consent Form (English Version)

Identification No: _____ Date: _____

Health Institution: _____

A study will be conducted under the objective of characterizing genetic diversity of malaria parasites in Ethiopia. For this purpose, a blood sample will be taken by finger pricking. A microscopic diagnosis will be done on the same specimen to detect malaria parasites and the rest of the specimen will be taken by the investigator. The collected blood specimen might be used for different research purposes; if needed it might be sent abroad for another related study.

Patients who are above three months of age, suspected to have malaria and who consent to participate will be included in the study.

To avoid infection with blood borne parasites like HIV and HBV, one disposable lancet will be used for finger pricking each patient. For those who have bleeding problem, special care will be given.

All costs related to the microscopic examination and antimalarial drugs will be covered by the investigator.

I, who registered in _____ identification number, clearly understood the above statement and agreed to participate in the study.

Name and Signature of the participant/parent/care-taker

_____ _____

Appendix C Consent Form (Amharic Version)

የወባ ተውሳክ ዝርያ ባህርይ ላይ የሚደረግ ጥናት

የፈቃደኝነት መግለጫ ቅፅ

መለያ ቁጥር _____ ቀን_____

የጤና ተቋም_____

የወባ በሽ አምጭ በሆኑት ጥቃቅን ጥገኛ ህዋሳት ላይ ጥናት ለማዘጋጅ ቅዷል። የጥናቱ አላማም በነዚህ ህዋሳት ዘረ-ህዋስ የሚየውን የዘርያ ተላዋዋጭነት ማጥናት ነው። ለዚሁም ተግባር የደም ናሙና ከጣት ጫፍ በምላጭ በመብጣት ይወሰዳል። የወባ በሽ ለመሆኑ ማረጋገጫ የሚገኝበት የላብራቶሪ ምርመራም የሚደረግው በዚሁ ጊዜ ከሚወሰደው ናሙና ይሆናል። የተቀረው ናሙና ግን ለምርምር የሚወሰድ ይሆናል። የዚህ ጥናት ተሳፊ ሊሆኑ የሚችሉትሩ ዕድሜአቸው ከ3 ወር በላይ የሆኑ«የወባ በሽ ምልክቶች የሚየባቸውና የደም ናሙና ለመስጠት ፈቃደኛ የሆኑ ናቸው።

በደም ናሙና አወሳሰድ ወቅት ሊከሠቱ የሚችሉ ችግሮች ንደ ኢንፌክሽን ና የደም አለመርጋት የመሳሰሉትን ለማስወገድ ለእያንዳንዱ ሰው አንድ የመብጫ ምላጭ የምንጠቀም ሲሆን የደም አለመርጋት ችግር ላለባቸውም ከፍተኛ ጥንቃቄ ይደረጋል። የደም ናሙና ምርምራና የፀረ ወባ መድሃኒት ወጪ በአጥኑው ቡድን ይሸፈናል ። የተወሰደው ናሙና እና ከውስጡ የሚገኘው የወባ ተውሳክ ዘር ለተለያየ ጥናት ኋለፈለገም ከአገር ውጪ ተልኮ ሊመረመር የችላል ።

እኔ በ_____ መለያ ቁጥር የተመዘገብኩት ግለሰብ ከላይ የተጠቀሰውን ሃሳብ ተረድቼ በጥናቱ ተሳፊ ለመሆን ፈቃደኛነ ን በፊርማ አረጋግጣለሁ።

የተሳፊ ወይም የተሳፊ ወላጅ / ጠባቂ/ ስምና ፊርማ

ስም_____ ፊርማ_____