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DEPARTMENT OF MEDICAL LABORATORY SCIENCES

Genetic diversity of *Plasmodium falciparum* field isolates based on two PCR markers Merozoite Surface Protein 1 and 2 from kolla-shelle area, Arbaminch Zuria district, South West Ethiopia

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A thesis submitted to the Department of Medical Laboratory Sciences, School of Medicine, College of Health Science, Addis Ababa University, in partial fulfillment of Master Of Science Degree in Clinical Laboratory Sciences (Diagnostic and Public Health Microbiology)

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ADDIS ABABA UNIVERSITY COLLEGE OF HEALTH SCIENCE SCHOOL OF MEDICINE

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List of abbreviations

AAU	Addis Ababa University
ACT	Artemisinin-based combination therapy
bp	Base-pair
DBS	Dried Blood Spot
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylene diaminetetraacitic acid
EPHI	Ethiopian Public Health Institute
FMOH	Federal Ministry of Health
Hb	Hemoglobin
IAEA	International Atomic Energy Agency
IPT	Intermittent Preventive Therapy
LLIN	Long Lasting Insecticide Treated Nets
ma.s.l	Meter above sea level
MOI	Multiplicity of infection
MSP	Merozoite surface protein
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
SNNPR	Southern Nation Nationality Peoples Region
TAE	Tris-acetate buffer-EDTA
WHO	World Health Organization
µl	Microliter
UV	Ultraviolet

Abstract

Background: The population structure of the causative agents of human malaria, *Plasmodium* species including the most serious agent *Plasmodium falciparum (P.falciparum)*, depends on the local epidemiological and demographic situations, such as the incidence of infected people, the vector transmission intensity and migration of inhabitants (i.e. exchange between sites).One of the major characteristics of malaria parasites is their genetic diversity and an increasing number of studies have been reported on the population structure variation of *P. falciparum* based on the polymorphism of merozoite surface protein (MSP) 1 and 2. Limited data however are available from Ethiopia.

Objective: To evaluate the extent of genetic diversity of P *falciparum* in Kola-Shele in South West of Ethiopia.

Methods: Health facility based cross sectional study design was employed to determine the prevalence of genetic diversity of *P.falciparum* in Kola-Shele area. Eighty-eight stored dried blood spot samples which were collected between September and December, 2008 were used. Parasite DNA was extracted from the blood spot on to filter paper and analyzed by length polymorphism following gel electrophoresis of DNA products from nested polymerase chain reactions targeted block 2 of *msp-1* and block 3 of *msp-2,*including their allelic families: K1,MAD20,RO33 and FC27,3D7/IC1, respectively. Data entry was done using Microsoft Excel sheet and was double entered to verify accuracy; data was analyzed using SPSS for windows 16 soft ware (SPSS INC, Chicago, IL, USA).

Results: The total number of alleles identified in MSP1 block 2 was 11, while 12 alleles were observed in MSP2 block 3.In MSP1, K1was found to be the predominant allelic type, carried alone, with MAD20 and RO33 type. In MSP2, 3D7/IC was the most identified. Forty- three and

sixty nine percent of isolates MSP1 and MSP2, respectively had high multiple genotypes and the overall mean multiplicity of infection was 1.8 (95% CI: 1.48-2.04).

Conclusion: The Genetic diversity in *P. falciparum* field isolates in kolla-Shelle area were mixed and multiple infections were observed. K1 and 3D7/IC1 were the most predominant circulating allelic families.

1. Introduction

Malaria is a protozoan disease transmitted by the bite of infected female Anopheles mosquitoes. *Plasmodium falciparum* (*P. falciparum*) causes more than 200 million cases of malaria each year, with greater than 1 million deaths (1). Widespread implementation of artemisinin-combination therapy and long-lasting insecticide bed nets (LLIN) have been linked temporally to declines in the incidence of malaria in certain areas of Africa (2); however, such gains are constantly threatened by the emergence of drug-resistant *P. falciparum* parasites and insecticide-resistant mosquito vectors (3,4). Malaria is a leading health problem in Ethiopia. About two-thirds of the population lives in malarious areas (5).

P falciparum is the most virulent of the five parasite species inclusive of *P. knouwlesi* which cause malaria in humans (6). This malaria parasite is genetically diverse at all levels of endemicity. This diversity variation provides mechanism for the survival of the parasite. Thus, typing of the genetic variation in malaria parasites is expected to provide new insights for the deployment of control measures (7).

P. falciparum exhibits a high level of genetic diversity, which is manifested due to their polymorphic features, the merozoite surface proteins of the MSP-1 and 2 genes have been employed as polymorphic markers (7, 8). The most characteristic of human malaria parasites challenge is lack of license vaccine production to their genetic diversity and an increasing number of studies have been reported on the epidemiology of *P falciparum*, mainly focusing on the polymorphism of merozoite surface protein (MSP) 1 and 2 genes.

The merozoite surface protein 1 is an abundant protein on the surface of the merozoite first described by Holder and Freeman (9). MSP-1 of *P. falciparum* is a major surface protein, with an approximate molecular size of 190 kDa. It plays an important role in erythrocyte invasion by the merozoite (10). This gene contains 17 blocks of sequence flanked by conserved regions. Block 2, which is the most polymorphic of MSP-1 it grouped into three allelic families namely MAD20, K1, and RO33 type (11).

MSP-2 is glycoprotein consisting five blocks where central block is the most polymorphic (12). The MSP-2 alleles generally fall into two allelic types, FC27 and 3D7, which differ considerably in the dimorphic structure of the variable central region, block 3.

Many studies have used the polymorphic regions of MSP-1 and 2 as genetic markers to determine the diversity of *P. falciparum;* the multiplicity of infection, the level of malaria transmission, as well as to investigate the relationship of these factors with acquisition of natural immunity against malaria (13, 14). Moreover, these marker proteins are immunogenic in humans and have been identified as potential candidates for blood-stage malaria vaccine (14-16). Identifying *falciparum* malaria population diversity in relation to these markers verses eco-epidemiological distribution, variation in dominance pattern and its correlation with disease severity is of paramount importance for future vaccine trials in Ethiopia. This study was under taken to evaluate the extent of genetic diversity based on isolates of *P. falciparum* collected from a health facility in Kolla-Shele area South West of Ethiopia.

1.1. Biology of Malaria

Malaria is a major disease of humans caused by a single celled protozoan parasite of genus *Plasmodium;* transmitted by mosquito of the genus *Anopheles. P. falciparum* is the most virulent of the five parasite species.

P. falciparum is generally confined to tropical and subtropical regions because the development of the parasite in the mosquito is greatly retarded when the temperature falls below 20^0 C. *P. falciparum* causes severe malaria because it multiples rapidly in the blood, and can thus cause severe blood loss (anemia). It has been suggested that anaemia is a consequence of the destruction of parasitized and unparasitized erythrocytes and suppression or dysregulation of erythropoiesis In addition; the parasites can clog small blood vessels .When this occurs in the brain, cerebral malaria results as a complication that can be fatal.

The life cycle of *P. falciparum* involves the mosquito vector and human host (Figure 1). During a blood meal; a malaria-infected female *Anopheles* mosquito inoculates sporozoites into the blood vessels and through the circulatory system they are takento the liver.each sporozoites

differentiated and replicates asexually to give rise to thousands of merozoites. Each sporozoite differentiates and replicates asexually to give rise to thousands of merozoite forms of the parasite. Membrane bound merosomes that contain the exoerythrocytic merozoites then bud off from hepatocytes into the liver sinusoids (17), which later rupture and release merozoites into the blood stream.After infecting red blood cells. Merozoites develop into mature and into schizonts which rupture releasing merozoites .Some parasites however differentiate into sexual erythroytic stages (gametocytes)., that further differentiate into micro(gametocytes) and (macro)gametocytes,when ingested by an *Anopheles* mosquito during a blood meal..The microgametes penetrate the macrogametes generating zygotes into mosquito stomach. The zygotes in turn become motile and invasive elongated ookinetes which invade the midgut wall of the mosquito where they develop into oocysts. The oocysts grow, rupture, and release sporozoites , which make their way to the mosquito's salivary glands. This parasites multiplication in the mosquito is known as the sporogonic cycle Inoculation of the sporozoites into a new human host continues the malaria life cycle.

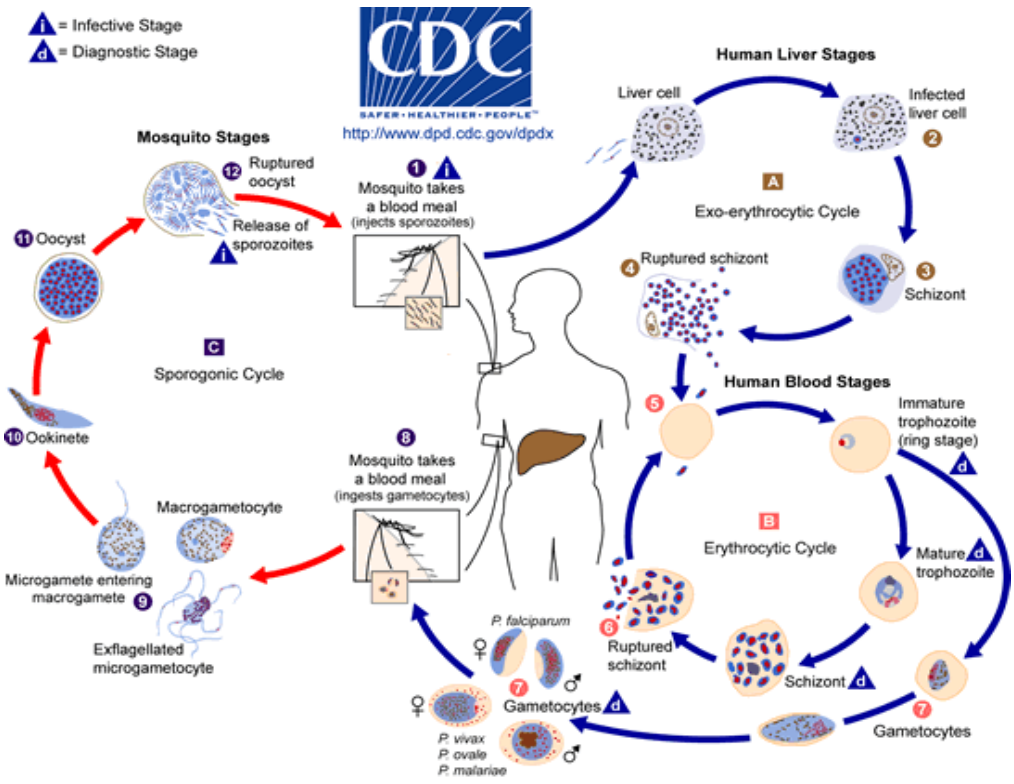


Figure 1. The life cycle of Malaria parasite (Source: CDC, 2010)

1.2 Statement of the problem

Malaria is s a serious problem in Ethiopia where the country's economy is based on agriculture; and peak malaria transmission coincides with the planting and harvesting season, this has placed a major economic burden on the country (22).

Problem of malaria control is becoming difficult in our time because of development of resistance to commonly use anti-malarial in parasites, to insecticides in vectors and operational problems. Therefore, to develop suitable and novel control strategies against the parasite, it is important to know the extent of genetic polymorphism existing in the parasite population. In Ethiopia, the results of 2008 efficacy monitoring study showed 7.5% treatment failure rate (46) to the first line Arthemisin combination Therapy (ACT) ,Artthemether-lumefatrine (Coartem).

Genetic variability of the *Plasmodium* parasites has made them successful against all the eradication efforts made by the human. Historically, vaccines have been one of the most cost-effective and easily administered means of controlling infectious diseases, yet no licensed vaccines exist for malaria.

In malaria-endemic regions, *P. falciparum* infection is characterized by extensive genetic diversity. Describing this diversity provides important information about the local epidemiology of malaria and is crucial to understand the parasite population structure and virulence, and for evaluating the impact of malaria control measures.

Recently ,some strains of *P.falciparum* has been reported to have developed resistance to ACT in South East Asian countries .In Ethiopia evaluation of therapeutic efficacy study results in 2008 , showed that there is 7.5% treatment failure rate in Kolla-Shele area, Arbamench Zuria district, South West Ethiopia. This finding is alarming because it is very high compared to other similar studies in Ethiopia. This result prompted us to conduct a genetic diversity study with two highly polymorphic markers (*msp 1* and msp *2* genes) from the previous pre-treatment (Day 0) stored blood spot samples. There is limited report available on the genetic diversity of *P. falciparum* populations in Ethiopia. The current finding on molecular genotyping will characterize the circulating allelic types of *P. falciparum* population.

2.1 Global Malaria Situation

The risk of malaria is highly dependent on interactions between the human host, the parasite and the mosquito vector. The causative agent of malaria is a single-cell protozoan parasite, widespread in tropical and subtropical regions, including parts of the Americas, Asia, and Africa. Malaria is one of the most severe public health problem worldwide. It is a leading cause of mortality and morbidity in many developing countries, where young children and pregnant women are the groups most affected. Half of the world's population (3.4 billion people) lives in areas at risk of malaria transmission (18).

Among the malarial parasites, *P. falciparum* causes the most severe malarial attacks and is responsible for the high morbidity and mortality (19).Africa accounts for the majority of estimated malaria cases (80%) and deaths (90%) (20). Recent data, however, indicates that effective programs reduce newly reported cases by at least 75% in eight countries (Botswana, Cape Verde, Eritrea, Namibia, Rwanda, Sao Tome and Principe, South Africa, and Swaziland) (20).

There were 97 countries with ongoing malaria transmission in 2013, and approximately half the world's population is at risk for infection, worldwide. According to WHO, 2013 malaria report, there were an estimated 207 million cases of malaria in 2012 and an estimated 627 000 deaths. Almost 90% of all malaria deaths occur in sub-Saharan Africa and of these 77% occur in non immune segment of population, mainly children under five years of age (Figure 2).

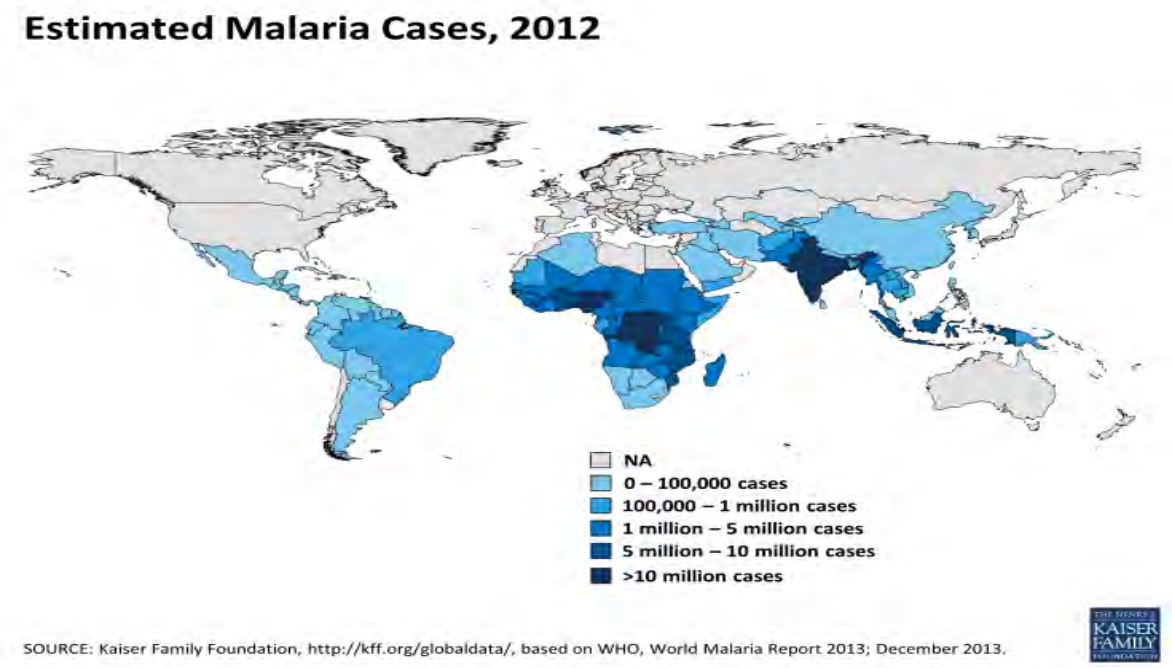


Figure 2: Global distribution of malaria cases, 2012**. (**Source: WHO, 2013)

This figure shows varying distribution of malaria cases ranging from nonmalarious areas highlighted in light gray (NA) to dark blue color that shows areas with high malaria cases transmission (bottom squared key)

2.2 Malaria situation in Ethiopia

Malaria is a major public health problem in Ethiopia. Transmission is seasonal and largely unstable in character mainly due to the country's topographical and climatic features (rainfall and temperature) (21).

The epidemiology of malaria in Ethiopia hence, differs from that in most of sub-Saharan Africa. Transmission is heterogeneous and generally lower than in other countries, and *P. vivax* is co-endemic with *P.falciparum* and causing up to 40% of clinical cases (22).

The two main seasons for transmissions of malaria in Ethiopia are between September and December, after the main rainy season, and the second malaria transmission period from April to May, following a short rainy season from February to March. There are four major eco-epidemiological strata of malaria: malaria free highland areas above 2,500 meter altitude, highland fringe areas between 1,500 and 2,500 meter which are affected by frequent epidemics,

and Lowland areas below 1,500 meters with seasonal pattern of transmission and low lands with stable malaria areas, characterized by nearly all year round transmission (23).

The 2007 Malaria Indicator Survey (MIS) indicated that parasite prevalence in Ethiopia was 0.7% and 0.3%, respectively for *P. falciparum* and *P. vivax* below 2,000 meters altitude. The 2011 MIS shows that 1.3% of all age groups were positive for malaria using microscopy and 4.5% RDTs using below 2,000 meters. *P. falciparum* constituted 77% of these infections. The 2011 MIS survey demonstrated a remarkable demarcation of malaria risk at an altitude of 2,000 meters, with thirteen-fold higher malaria prevalence at lower altitudes compared to higher elevations. There was essentially no *P. falciparum* detected by microscopy among persons surveyed within households having measured elevations above 2,000 meters in the 2011 MIS (23).

Despite the low malaria parasite prevalence compared to many African countries, malaria remains the leading communicable disease seen at health facilities in Ethiopia. Approximately 12 million suspected malaria cases are reported each year in Ethiopia. Malaria accounts for a total of 3,384,589 cases of whom 1,793,832 (53.0%) laboratory confirmed,(1,061,242 (59.2%) *P. falciparum* and 732,590 (40.8%) *P.vivax)*. Ethiopia reported 936(68%) malaria deaths in 2011, according to the 2012 World Malaria Report (23).

A small study showed a decline in the overall incidence of malaria accompanied by a shift from predominantly *P. vivax* cases to 73% *P. falciparum* cases (24). Recently however Coatem's higher differential lethality on *P. falciparum* than *P.vivax,* there has been a major shift from *P. falciparum t*o *P. vivax* dominance in different parts of the country. P. ovale (P. *ovale curtisii, P. ovale wallikeri.)* and P. malariae are also reported to occur (24). *Anopheles arabiensis* is the major malaria vector; *An. pharoensis, An. funestus a*nd *An. nili* are considered as secondary vectors, the last one more localized to South West Ethiopia (25).

2.3 Genetic diversity of *P. falciparum*

Many studies have been concluded on the epidemiology of genetic diversity of the parasite, mainly focusing on the polymorphism of msp-1 and msp-2 genes. Parasite populations have been

studied in different geographical areas with different transmission potential. Due to the combined allelic variation MSP1and MSP2 genes have been employed as a polymorphic marker in studies of malaria transmission dynamics in natural isolated of *P.falciparum* infection.

PCR-amplification of polymorphic regions of the two marker genes MSP1 and MSP2 were used to describe how genetic diversity and multiplicity of infection vary in accordance with age and parasite density. The allelic diversity of MSP2 has been observed to be high in the Côte d'Ivoire (27), whereas in Malaysia MSP-1 was the predominant one with RO33 identified in 80.0% and 3D7 76.0 % of samples (28). Another study in Senegal showed all MSP1 allelic variants MAD20, K1 and RO333 as markers *P.falciparum* malaria diversity , but their frequency varies in different geographical areas, even in neighboring villages' in areas of low malaria transmission (29).

Thus, many studies have used the polymorphic regions of MSP 1 and 2 as allelic markers to determine the genetic diversity of *P. falciparum*, multiplicity of infection, assess the level of malaria transmission, as well as investigating the relationship of these factors with acquisition of natural immunity against malaria (30,31). Areas of intense transmission are generally characterized by extensive parasite diversity, and infected individuals often carry multiplicity of infection (32,33). On the other hand, the parasite population in low transmission areas has limited genetic diversity and most infections are monoclonal (34,35).

Since protection immunity specificity and avidity is both species strain dependent, genetic diversity has been reported to play key role in the acquisition of anti-malaria parasite immunity (32,37,38).Therefore, identifying the genotypes circulating in different geographical locations would facilitate the development of effective control strategies like vaccine. But, the inherent variability of *P. falciparum* as observed in merozoite surface antigens provides multiple effective evasion mechanisms for the parasite. It thus represents a major challenge for development of an effective malaria vaccine. Hence, the study of genetic diversity in malaria parasites is expected to provide new insights for the deployment of control measures.

A major mechanism for the generation of allelic diversity in the *P. falciparum* is due to meiotic recombination in the *Anopheles* mosquito, which is believed to be dependent on the intensity of transmission. It is suggested that frequent recombination events as in MSP-1 alleles intermittently generate novel alleles in high transmission areas (33).However, the use of only one or two markers, no matter how polymorphic they are, would miss variation at other polymorphic loci, and thus, almost certainly underestimate the magnitude of multiple infections. Thus incorporating other markers like microsatellite DNAs would give a better picture. Nevertheless, the choice of a particular gene marker for typing natural *P. falciparum* clones depends on the question being addressed.

2.4 Malaria prevention and control

According to the WHO recommended approaches the control of *P. falciparum* malaria are broadly classified into case management and prevention (39). Prevention efforts includes prompt diagnosis and treatment, prophylactic antimalarial drugs, IPT and mosquito-control activities to prevent infection. The mosquito-control activities are broadly classified as; vector control by means of indoor residual spraying (IRS), insecticide treated nets (ITN) and larval control besides repellants and other personal and house hold protective measures.

The malaria prevention and control program in Ethiopia has developed several national statgies. The current national strategy (2015-2020) has the following major components besides the epidemic surveillance and health facility development (41)

Component 1: Community Empowerment and Mobilization

Community empowerment and mobilization are central to malaria prevention and control. Ethiopia's Health Extension Program educates, mobilizes and involves the community in all aspects and stages of malaria control and leads to increased ownership of the program.

Component 2: Diagnosis and Case Management

Since 2005, there has been a major shift from clinical diagnosis to confirmatory diagnosis following the wide-scale use of RDTs in health posts. The objectives of this component are: 1) 100%of suspected malaria cases are diagnosed using RDTs and or microscopy within 24 hours of fever onset; 2) 100% of positive malaria diagnoses are treated according to national guidelines and 3) 100% of severe malaria cases are managed according to national guidelines.

Component 3: Prevention:The main major vector control activities implemented in the country include IRS, LLINs and environmental control. The objective of this component is to ensure that 100% of households in malarious areas own one LLIN per sleeping space, and that at least 80%of people at risk of malaria use LLINs and IRS is currently targeted to cover epidemic prone areas and malaria affected communities with low access to the health care system.

3. Objectives of the study

2.1. General objective

To describe the genetic diversity of *P falciparum* in Kolla Shelle area, South West Ethiopia.

2.2. Specific objectives

- To determine genetic diversity of *P. falciparum* isolates from Kolla Shelle area, Arbaminch district using merozoite surface protein-1 and 2 (MSP-1 & 2).
- To determine the prevalence of allelic diversity of msp genes in blood collected from malaria patients attending health center.

4. Hypothesis of the study

There is a moderate genetic diversity expected from samples collected from Kola-Shele area South West Ethiopia.

5. Materials and methods

5.1 Study design

It is a cross sectional health facility-based study, carried out on stored dried blood spot (DBS) The source of the samples were collected from patients visiting Kolla-Shele health center and enrolled in to the study for evaluating the therapeutic efficacy of Coartem® in the treatment of uncomplicated *P. falciparum* malaria between September and December in 2008, in Kolla-Shele area, South West, Ethiopia.

5.2 Study site

The study samples (DBS) were collected from patients attending Kolla-shele health center for coartem efficacy study. Kolla-shele health center is located about 27 kms from Arbaminch town and 532 kms from Addis Ababa; Kolla Shele is one of the kebeles of altitude Arbaminch Zuria ditrict , with a catchment population of 47044 inhabitants (Figure 7).This area lies at an between 1250 and 1400 meters above sea level (asl). Malaria transmission is highly seasonal, and markedly unstable. The study area has an entomologica inoculation rate (EIR) of 17.1 infectious bites per person per year (45) .Malaria infection is primary due to *P.falciparum.*

Subsistence farming and fishing are the main occupation in the area. Water for mosquito breeding is available throughout the year because of rivers Sille, Sego, and Lake Chamo.

Arbaminch Zuria is one of the 77 Districts in the SNNPR, Ethiopia. The total population of Arbaminch Zuria ditrict was estimated to be 189,810 in 2003 E.C. (2010/2011), 93,069 males and 96,803 females. It is a part of the Gamo Gofa Zone situated in the Western Great Rift Valley with an altitude ranges between 1200-3125 m asl. The District receives an average rainfall from 750mm^3 to 930 mm^3 and mean annual temperature ranges between 16^0 C and 37^0 C (43).

5.3 Study period

The molecular analysis of genetic diversity of *P.falciparum* was conducted at EPHI malaria molecular laboratory between January and May 2014, on stored dried blood spot (DBS).

5.4 Population

5.4.1 Study population

DBS was collected from patients who visited the health centers during the evaluation of the therapeutic efficacy study , September-December, 2008. Those patients that consented and were febrile, ≥37.5 axillary temperature and positive for asexual *P. falIparum* and are residents within the catchment area (i.e. 5-10 km radius of the health center) were enrolled, in accordance with the inclusion criteria (44).

5.4. 2 Sample size determination

A total of 88 *P falciparum* infected blood spot samples collected for drug efficacy monitoring study were used to evaluate genetic and allelic diversity of *P. falciparum*. Patients who participated in the drug efficacy and monitoring study were within the age range of 6 months to20 years and selected following the WHO protocol (45).

5.4.3 Sampling procedures

Patients attending the health facility were initially screened for the lowest limit of acceptable enrollment parasite density. This was performed by microscopic examination of rapidly stained thick film for the presence of at least one parasite (*P. falciparum*) against 6-8 WBC, corresponding to 1000/µl asexual parasitemia. Once the patient satisfies the initial thick film screening, the patient's consent was requested and referred to the study team for further screening. Based on physical and clinical examinations patients any danger signs or symptoms were associated with severe malaria were excluded from the study and referred to emergency treatment ward.

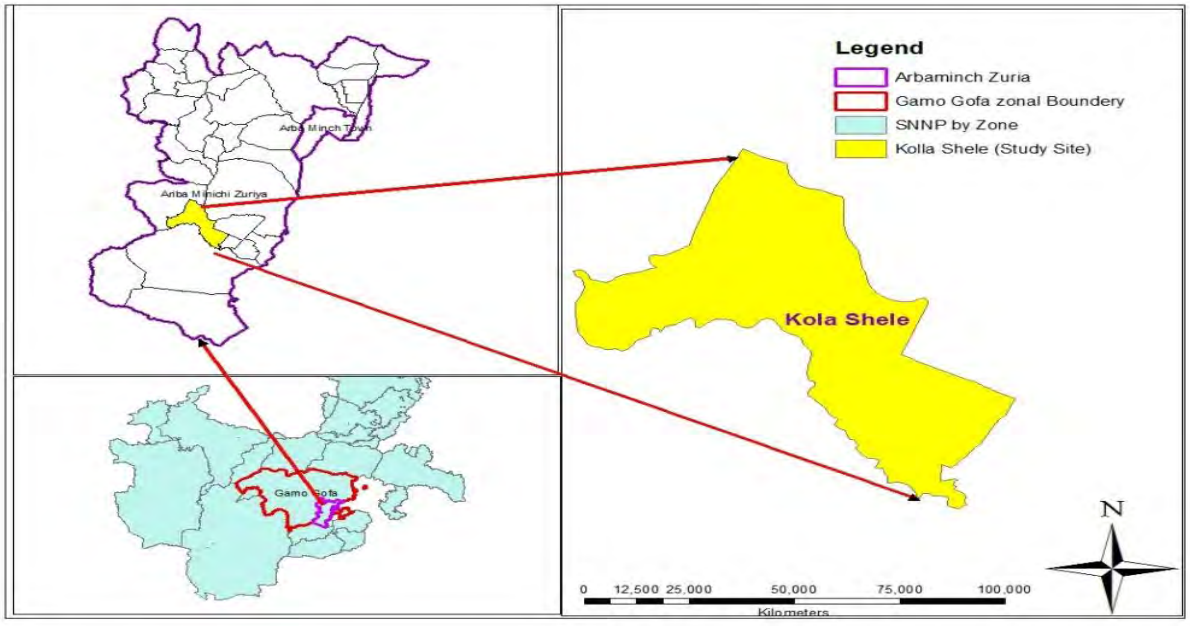


Figure 3: Map of Arbaminch Zuria showing location of the study site. (Source: CSA, 2007)(40).

5.4.4 Inclusion and exclusion criteria

5.4.4 .1 Inclusion criteria

The inclusion criteria of participant were: axillary temperature ≥ 37.5°C measured with an digital thermometer, malaria parasites (*P. falciparum)* in thick blood films (1000 - 100,000 parasites /µl). Informed consent was obtained from adult patients and from parents or guardians for children. Additionally, at inclusion into the study and before treatment, three drops of blood from each patient where blotted on Whatman® 927 mm filter paper (45). The filter paper was allowed to air dry and placed individually in plastic bags and stored at -20 ^{0}C with a silica gel (Sigma) to prevent dampness.

5.4.4.2. Exclusion criteria

Patients with severe signs of anaemia according to WHO criteria (haemoglobin < 5 g/dl), history of allergic reactions to the study drug Coartem®. Severe malnutrition and pregnant and lactating women were not included in the study (45).

5.5 Study Variables

5.5.1 Dependent variables

MSP, MSP2 genotype diversity and multiplicity of infection.

5.5.2 Independent variables

Age, gender, and parasite density of patients,

5.6 Measurement and Data collection

Information collection during these interviews included socio-demographic characteristics (age gender, patient used bed net and previous malaria attack), the dry blood spots were stored at 20^{0}C until molecular analysis performed by using WHO guideline.

5.6.1 Data Quality Control

Individual data points and laboratory data were independently entered into a database. The amplification of DNA by PCR was done independent of the microscopy results. Before analyzing the data they were cross-checked. Each molecular analysis was conducted in triplicates to minimize errors.

5.6.2 Data analysis and interpretation

The data was entered in Microsoft Excel sheet and double entered to verify accuracy; data was analyzed using the SPSS soft ware version 16 (SPSS INC, Chicago, IL, USA). The *msp-1* and *msp-2* allele frequency was calculated as the proportion of allele found for the allelic family out of the alleles was detected in isolates. Arithmetic means and medians were also calculated for all continuous baseline demographic variables, except for asexual parasite density (geometric mean). Furthermore Spearman's correlation coefficient, nd X^2-test were calculated to see association between age, parasite density and multiplicity of infection. P-value less than 0.05 was considered to be statistical significant.

5.6 Operational Definition

Anopheles*:* the genus of mosquito that transmits malaria. This genus includes many species that are vectors for malaria transmission.

Endemic: area where the diseases is constantly present.

Multiplicity of infection: the mean number of detected *P. falciparum* genotypes per infected individual.

Merozoite: the form of the malaria parasite that invades human red blood cells.

Merozote surface protein: is a glycoprotein expressed on the surface of merozoites, the stage of parasite that invade the RBCs

Polymorphism: genetic difference between organisms of the same species

Genetic diversity: genetic difference between organisms of the same species polymorphism and the difference accumulate between species

Drug resistance: is the ability for an organism to survive concentrations of a particular drug given at doses usually toxic to the organism.

Allelic: allele is one of two or more versions of a gene or variation among non-coding DNA sequences.

Strain: a genetic variant within a species

5.7. Laboratory procedures

5.7.1 Sample collection

Finger-prick blood samples were collected from consenting patients for malaria parasite identification and hemoglobin level measurement. Patients that satisfied the criteria were enrolled to the study on day 0 pre-treatment where finger-prick samples were taken for microscopic glass slides for preparation of thick and thin blood films were stained with 10% Giemsa, (pH= 7.2, for 10 minutes), while thin smears were fixed in methanol prior to Giemsa staining. Each blood smear were examined by experienced laboratory technician at the health centers and then re-checked by certified laboratory technician at Ethiopia Public Health Institute (EPHI)(former EHNRI). Parasite load were also calculated after counting asexual parasites per 200 white blood cells (WBC), assuming average WBC count is 8,000/µl (46). Parallel drops of blood were collected on filter paper (Whatman® 927 mm) on day 0 during enrollment. The filter paper was allowed to air dry and placed individually in plastic bags and stored at -20 ^{0}C with a silica gel (Sigma) to prevent dampness. Filter papers were transported to the laboratory of the Parasitology and Vector Borne Diseases Research team of the Ethiopian Public Health Institute for further molecular analysis.

Based on the microscopy results, the study subjects were classified into two groups: Group 1 that had hyperparasitaemia (total number of parasite >5000 parasites/µl of blood), group 2 that had hypoparasitaemia (total number of parasite ≤5000 parasites/µl of blood) (47).

5.10.2 Extraction of parasite DNA

From stored dried blood spots (collected on Day0) genomic DNA was extracted using Chelex-100 (Bio-Rad Laboratories CA) method; Excise the area of filter paper with the blood spot, and transfer the piece of filter paper to a sterile 1.5ml microfuge tube and incubated overnight at 4^0 C in 1 ml of 0.5% Saponine in 1x phosphate buffer saline (PBS).The filter paper was washed for 30 minutes in PBS at 4^0 C and the PBS was removed after incubation. Then, 50µl of 20% Chelex-100 solution to 150µl of DNase-free water was added directly to the tube and mixed for 30 seconds using vortexed. It was heated at 100 ^{0} C for 10 minutes to elute the DNA, and vortexes during the incubation. Then finally it was centrifuged at 10,000 rpm for 5 minutes the supernatant removed using a fresh tube the isolated DNA was stored at -20 ^{0} C until required for amplification (48).

5.7.6 Allelic typing of *P. falciparum* MSP-1 and MSP-2 genes

All samples were genotyped for *P. falciparum* using the nested polymerase chain reactions (PCRs) technique. The highly polymorphic loci was performed both for the variable block 2 regions of *msp-1* and block 3 of *msp-2*, considered to be the two most informative genetic markers for assessment of multiplicity of *P. falciparum* infection. The initial amplification was followed by individual nested PCR reactions using family specific primers for *msp-1* (KI, MAD20 and R033), and *msp-2* (FC27 and 3D7/IC1), respectively, based on published standard protocols (49).

The oligonucleotide primers specific for the polymorphic regions (block 2 of MSP-1 and block 3 of MSP-2) were designed as described previously (48). The two genes were amplified by nested PCR. An initial amplification of the outer regions of the two genes was followed by a nested PCR with family-specific primer pairs. All reactions were carried out in a final volume of 25 µl containing 20mM dNTP, 10µM of each primer, and 1 unit /25 ul reaction volume of Taq DNA polymerase (Roche Applied Science, Germany). In the first round reaction, 4µl of genomic DNA was added as a template. In the nested reaction, 1µl of the first PCR product was added. Each amplification profile consisted of initial denaturation at 94°C for 3 min, followed immediately by 30 cycles at 94°C for 1 min, 50°C for 35 sec, and 68°C for 2.5 min. The final cycle had a prolonged extension at 72°C for 3 min. PCR reaction mixtures were incubated in a thermal cycler ((MyCycler-BioRad, Hercules, USA)

Quality control

Allelic specific positive control (K1 and 3D7) and DNA-free negative controls were included in each set of reaction.

5.7.5 PCR analysis

The PCR product was separated by gel electrophoresis in 1xTAE buffer on 2% multipurpose agarose gel (Roche Diagnostic Gmbh USA) and visualized by ultraviolet transillumination after staining with ethidium bromide. Length of difference was determined using 100 base pair ladder. Five µ1 of nested PCR was mixed with a 1-2 µl of 10x loading dye and separated using 2% agarose gel ethidium bromide (8 µg /ml) in Tris-acetate buffer-EDTA (TAE) and DNA

visualized under ultraviolet (UV) trans-illumination. A100-base pairs (bp) DNA ladder marker was used to determine the size of band fragments. The size polymorphism in each allelic family was estimated assuming that one band represented one amplified PCR fragment, derived from a single copy of each gene.

5.7.6 Allelic distribution and multiplicity of infection

The prevalence of each allelic type was determined by the presence of PCR products for the type in the total number of amplified bands for the corresponding locus. The multiplicity of infection (MOI) or number of genotypes per infection was calculated by dividing the number of fragments detected in MSP1 or MSP2 by the number of samples positive for the same marker (50).

5.10.7 Ethical considerations

Scientific and Ethical approval was obtained from the Institutional Review Board (IRB) of Department of Medical Laboratory Science Addis Ababa University and Scientific and Ethical Review Office (SERO) of the Ethiopia Public Health Institute (EPHI).

6. Results

6.1. Demographic description

A total of 90 patients with clinical symptoms of malaria and microscopically confirmed P. *falciparum* infection that were enrolled in this study, *t*wo (2.2%) were exclude from the study due to unwillingness to participated in the study. Thus this work refers to the 88 consequence samples

Of these total positive samples, 51.1% were males and 48.9% were from females, with a mean age of 7.47 years and standard deviation of 5.3 years.

Patients belonging to under five years were the most represented with 47.7% (42/88) followed by those of 5-10 years of ages with 26.1 % (23/88), patients whose ages 10-15 years 18 (20.5%) and ≥ 15 years with 5.7 % (5/88).

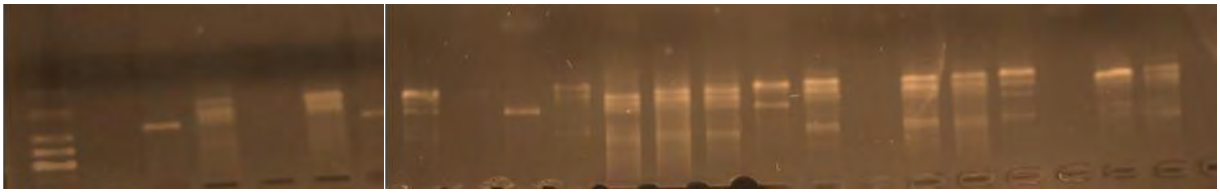
Table 1: Demographic characteristics of the study subjects *at* Kolla-Shelle health center, Arbaminch Zuria District South West, Ethiopia.(n=88).

Characteristics		Frequency	Percent (%)
Sex	Male	45	51.1
	Female	43	48,9
Age group			
	<5	42	47.7
	5-10	23	26.1
	10-15	18	20.5
	>15	5	5.7
Patient used bed net			
	Yes	36	40.9
	No	52	59.1
Previous malaria attack			
	Yes	74	84.1
	No	14	15.9
Parasite density			
	<5000	40	45.5
	≥5,000	48	54.5

The asexual P. falciparum parasitaemia from the collected samples ranged from 1000 to 100,000 parasites/µl of blood with a geometric mean was 7,701.5 parasites/µl. Low parasitaemic individuals (parasite count < 5000 parasites/µl of blood) represented 45.5%, while high parasitaemia (parasite count ≥5000 parasites/µl of blood) were 54.5%. Most children (91.1%) had previous experience malaria attack .Bed net use (46.7%) among children was not satisfactory (Table 1).

6.2. Allelic diversity size polymorphism

Using a 100 base pair DNA ladder molecular marker (MM) ,different fragments of base pairs of MPS1 and MPS2were identified by gel electrophoresis (Figure 5).



MM 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

Figure 4. Allelic family size polymorphism Using a 100bp DNA ladder molecular marker (MM) different fragments of base pairs of MSP2 were identified by gel electrophoresis.

This figure showed for FC27 lane 1 and 4 samples were negative, lane2, 320 bp lane 3, 420bp ,lane 5,500 bp ,lane 6,350 bp and 3D7/IC1 allelic type base pairs from lane8,16,20 were negative , lane 7, 520 bp, lane 9 ,350,lane 10,500bp,lane11,420bp, lane 12-19,21,22,were 400 bp.

6.3. Allelic diversity o*f P .falciparum* MSP1 and MSP2

Length polymorphism was assessed in 88 *P.falciparum* field isolates within the allelic families of *msp-1* and *msp-2,* with a total of 155 distinct fragments detected. This Allele typing analysis displayed the highly polymorphic nature of *P. falciparum* in Kolla-Shele isolates with respect to MSP-1. In MSP-1, K1, RO33 and MAD20 allele types were identified. Frequencies of different msp-1 alleles and their combinations and multiplicity of infection are shown in the Table 2.

Table 2: Allele typing and diversity profiles of *P. falciparum* isolates from southern Ethiopia based on genetic diversity of MSP-1, Koll-Shele area South west Ethiopia.2014

Allelic type	PCR product size (bp)	Frequency	Percent (%)
MSP-1			
K1	200-250	20	33.9
MAD20	100-300	5	8.5
RO33	150-225	9	15.2
K1 + MAD20			
RO33+K1		25	42.4
RO33+MAD20			
Mad20+K1+Ro33			
Total		59	100

The proportion of K1, MAD20 and RO33 types were 33.9%, 8.5% and 15.2 % respectively. The remaining nearly 42.5% were the allelic types of msp1 (K1/MAD20, K1/RO33, MAD20/RO33 and K1/MAD20/RO33).

The total number of *MSP1* different sized alleles detected in this sample was 11. Among them, three for K1 (200-250 bp), five for RO33 (150-250 bp) and three for MAD20 (100-300bp) allele families were observed (Table 3).

The monoclonal infections were 34 (57.6%). Among Polyclonal infections those that carried two allelic types K1/RO33, K1/MAD20, MAD20/RO33 comprised 25.4%, 5.1% and 3.4 % , respectively carried two allelic type . Trimorphic infections K1/MAD20/RO33were detected in 5.7% of cases.

Table 3: *Plasmodium falciparum* genotypes and base pair ranges observed in *msp-1* and *msp-2*, as well as their respective allelic families

	msp-1			***msp-2***	
	K1	**MAD20**	**RO33**	**FC27**	**3D7/IC1**
Base pair range	200-250	100–300	150-225	300-600	200- 500
No. of genotypes	3	3	5	7	5
Total no. of genotypes		11		12	
Overall multiplicity of infection		1.7		1.6	

Overall, the mean multiplicity of infection was 1.8 (95% CI: 1.48-2.04) of 88 samples 52 (59%) harbored more than one parasite genotype. when considering msp-1 and msp-2 genes separately, the mean multiplicity of infections were 1.7(95% C.I: 1.5-1.9) and 1.6 (95% C.I: 1.5-1.8), respectively .

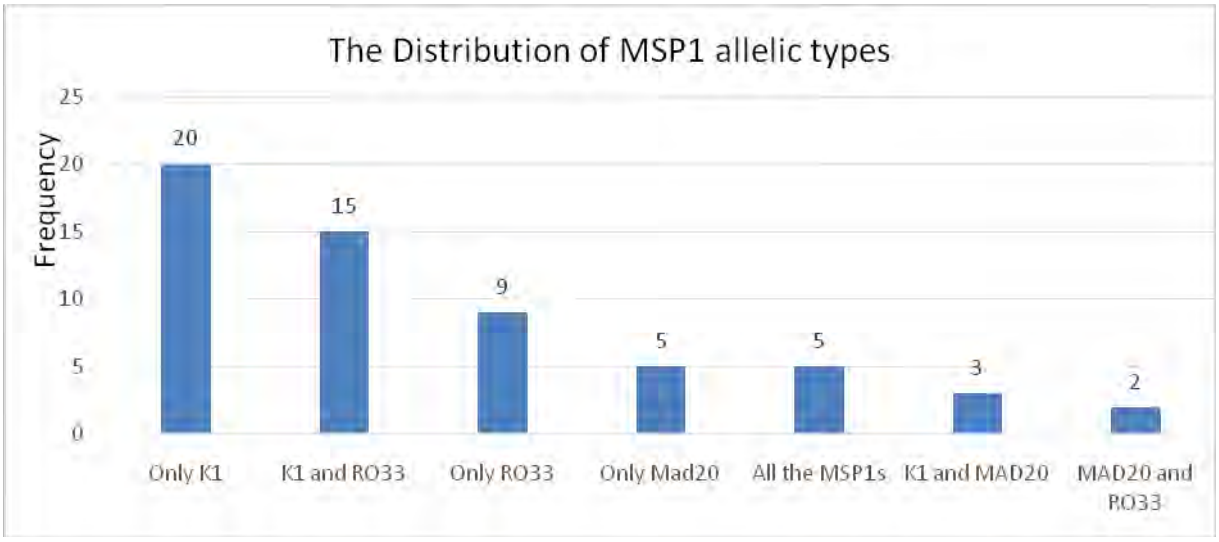


Figure 5.The distribution of MSP1 and their respective allele in Kolla-Shele area South West Ethiopia, 2014(n=88).

In *MSP1*, a total 11 different alleles were identified by size differences on agarose gels representing, MAD20 (3 alleles), K1 3 (3 alleles), and RO33 family (5 alleles) as showed Table 3. The frequencies of these alleles based on PCR products size (base pairs, bp) and their combinations are shown in Figures 5.

For MSP-2, both FC27and 3D7/IC1 allele types were identified among the isolates. The frequency of samples having only FC27 allele type was 10 % (4/39), while the frequency having only 3D7/IC1 allele type was 21 % (8/39). Twenty-seven of the isolated (69%) carried both *MSP-2* allelic families (Figure 6). On the other hand, monoclonal infection was detected in 31% (12/39) of cases were positive for *MSP-2* alleles while 69% were polyclonal infections.

The length variants of the amplified product were about 300-600 bp for FC27 and 200-500 bp for 3D7/IC1 (figure 5). A large proportion of isolates (59/88 for *MSP-1* and 39/88 for *MSP-2*) showed more than two PCR products for each locus, as visualized on agarose gel as a double band or multiple bands.

These results collectively suggest that diverse allelic polymorphism of *MSP-1* and *MSP-2* was identified in *P. falciparum* isolates from Kolla-Shelle area and that most of the infections were mixed.

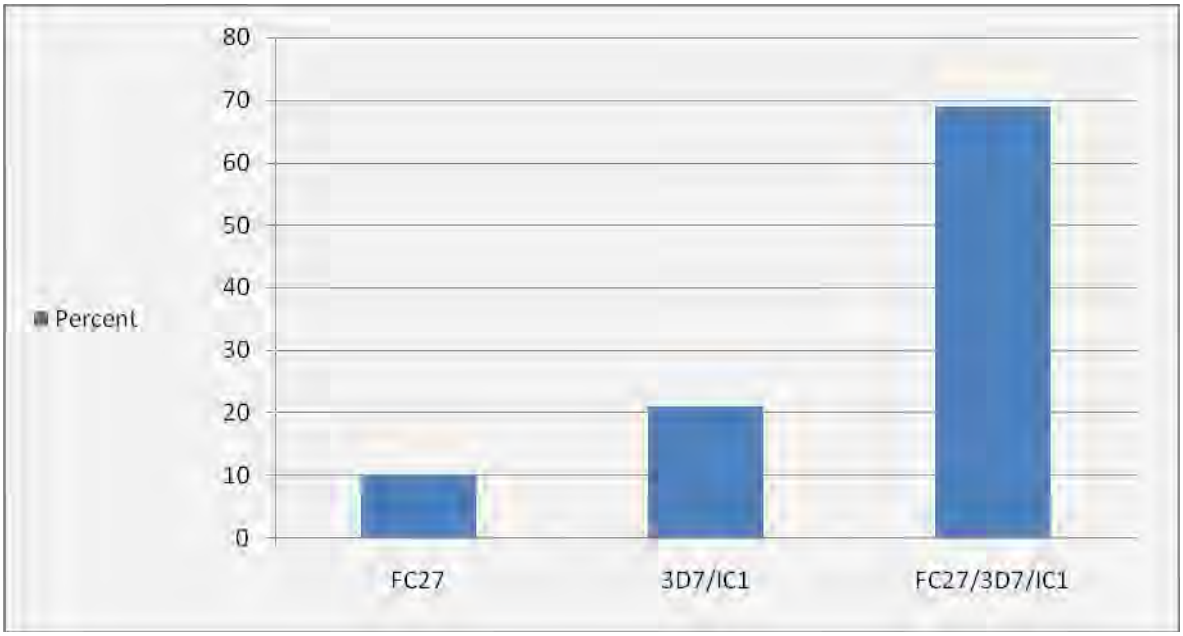


Figure6.The distribution of MSP2 alleles in Kola-Shele area, South West, Ethiopia 2014(n=88)

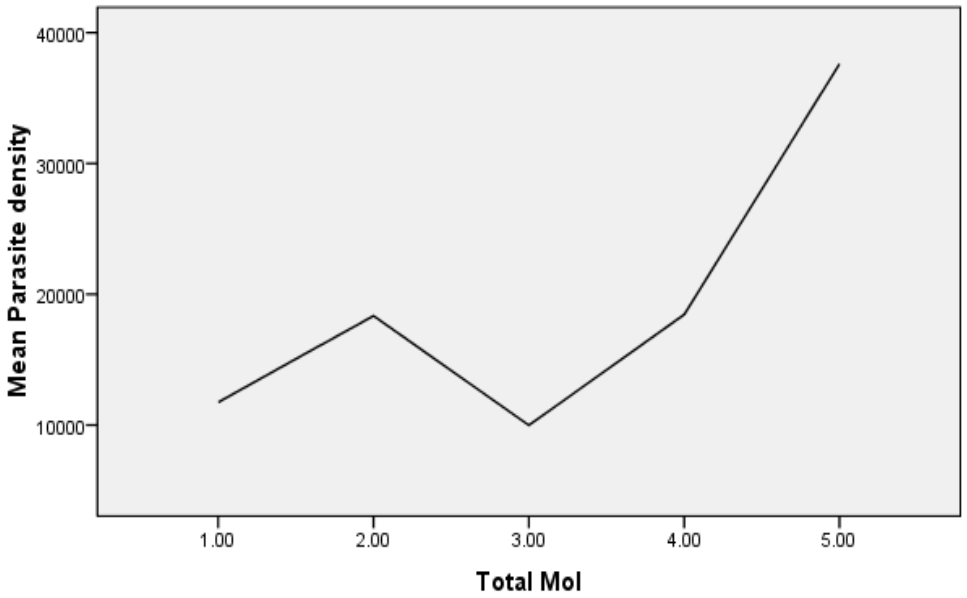


Figure 7. Relationship between mean parasite density and multiplicity of infection (n=88).

There was no significant correlation between multiplicity of infection and parasite density of patients (Spearman rank coefficient = 0.03; p = 0.8) was noted.

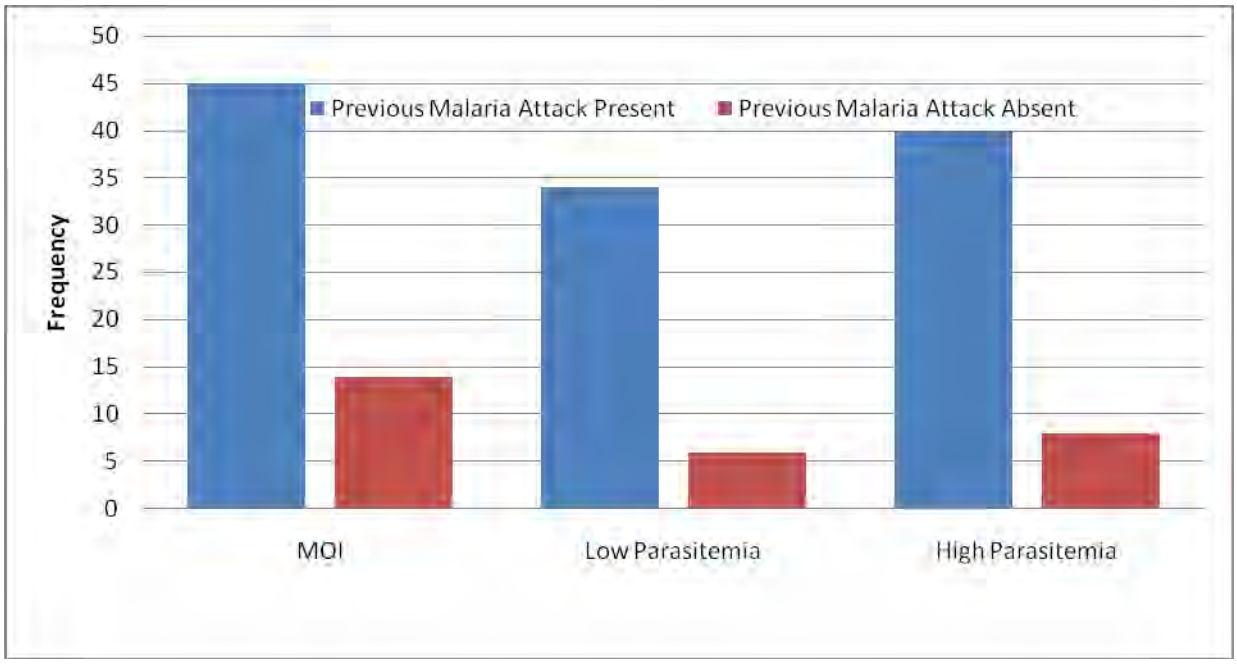


Figure 8 Comparison of MSPs and Parasite density against presence of previous malaria attack in the Kolla-Shell South West Ethiopia.

The previous malaria attack had high parasite density and multiplicity of infection (MOI) as showed in figure 8. Previous malaria attack (χ^2= 40.9, df=1, P=0.00)) have relationships with MOI(χ^2 =27.2,df=5, P=0.00) where as previous malaria attack have not significant relationship parasite density.

7. Discussion

Genetic diversity has been reported to play a key role in the acquisition of anti malaria immunity (32). A better understanding of population structure of P. falciparum genotypes may be an important element for implementing malaria control strategies in the country. In this study, genetic polymorphism of two merozoite surface protein, msp-1 and msp-2 of 88 *P falciparum* collected in Kolla-shelle area where malaria is endemic was analyzed. No such study has been done in Ethiopia to date, and therefore this study provides the first estimate of genetic diversity circulating at least in this study area.

The allele specific *MSP1* and *MSP2* genotyping data revealed moderate allelic diversity in *P. falciparum* isolates in the study area. The genomic DNA of the *P. falciparum* isolates were investigated for genetic diversity of at high polymorphic region of merozoite surface protein 1 and 2 genes. The MSP1 and MSP2 regions have been genotyped using the nested polymerase chain reaction (PCR) techniques and conditions previously described.

We observed the overall mean multiplicity of infection was 1.8. Regarding of the Msp2 gene, alleles belonging to 3D7/IC1 family were mostly detected, both in mono-infection and mixed infection with FC27alles. This finding is consistent with the situation in Kenya and Peru (10,55)

Regarding of MSP1 gene, K1 allelic family was predominant, consistent with the previous studies in Gabon and Central Africa (16,49). RO33 alleles were more prevalent in Uganda (49). The current parasite profile with the predominance of K1 and 3D7/IC1 was also presented from patients with uncomplicated *P.falciparum* malaria in Gabon (16).

Our findings showed a high multiplicity of infection (MOI) for both MSP-1 and MSP-2 reflecting the high intensity of malaria transmission in the study area and this is in agreement with previous observations of an increased MOI with increasing endemicity (10, 11).

A EIR study conducted in the same area showed a value of EIR 17.1 (infectious bites per person per year)(43), the moderate EIR value reported shows the presence of high parasite transmission this is in agreement with our finding i.e. high genetic polymorphism and MOI (59% and 1.8 respectively). The result is also in agreement to the Senegal situation where patients receive a large number of infective bites (28).

The frequency of the MSP-1 allelic families was higher than MSP-2. The predominant families in the MSP-1 and MSP-2 were K1 and 3D7/IC1, respectively. The results indicate that both K1 and 3D7/IC1 are good indicators for determination of MOI, due to high numbers of bands were encountered with these genes .these finding are different from Sudan and Uganda (15,52) which demonstrated the predominance of the RO3 3and FC27 allelic family. In this study, however we found that the K1 the most frequent allele .This results similar to previous studies done Malaysia and Ghana (10,49) but differs from study in Sudan (52), where RO33 was the most predominant

allelic family .This discrepancy might be due to different in the degree of transmission intensity or seasonal variability.

Regarding *MSP2*, the predominant allele type was 3D7/IC1; this is consistent with reports from the study in sub-Saharan-African (Tanzania, Burkina Faso, Malawi and Uganda) (53). The FC27 fragment of 400bp, which was the most prevalent in clinical episodes of malaria among symptomatic malaria children, similarly observation reported from the Sudan and Republic of Congo (52,57).This may be indicate an association between this FC27 allelic type and clinical episode. Hence this predicts a possible candidate antigen that may be considered in designing malaria vaccine.

In the current study, parasite density was found to be low in children aged less than 2 years and increase with age greater than 15 years. This study is different from previous studies done in West Uganda and Sudan (51,52). This could be explained by massive distribution of ITNs by the government and supporting agencies and deployment of ACT in the country; decrease of the burden of malaria parasites which is reflected in lower parasite densities, however this did not influence the diversity of parasites in circulation.

In this study, the number of parasite genotypes carried by subjects with symptomatic infections was not influenced by age, consistent the study of Republic of Congo (57).

The result of this study showed that mean multiplicity of infection of MSP-1 and MSP-2 genotype was 1.7 and 1.6, respectively. This finding was higher than the previous study in cote d'Ivore where the multiplicity of *P.falciparum* infection (MOI) was 1.37 and 1.20 for MSP-1 and MSP-2, respectively (26).This discrepancy is due to different geographical areas and transmission pattern.

In this study, we have reported that about two-third of the samples (59%) hardboard multiple genotypes, almost similar frequency patterns are observed in Sudan (62%) (52).

In our study, the frequency of FC27 and 3D7/IC1 allele type with (10 %, 4/39) and (21%, 35/88), respectively, but a higher proportion of isolates (69%, 27/39) contained both allele types. Similar

frequency pattern was observed Iran and Cameroon (13,54), but not in Sudan ,where FC27 type is more prevalent (52).These results collectively suggested that diverse allelic variation of MSP1 and MSP2 exist in *P .falciparum* in Kolla-Shelle area isolates and that most of the infections were mixed. This consistent observation was made in other endemic areas (10,16,52).

The mean *MOI* of persons with previous exposure to malaria attack is higher compared to persons with absence of previous malaria attack (non-exposed) i.e. high MOI correlates with high parasitic density (Figure 8). The finding may also indicate that persons with lower parasitic density may have low e acquired immunity (higher risk in clinical malaria).

8. Strengths and limitations of the study

8.1 Strengths of the study

This study provides information about the genetic diversity of *P. falciparum* in the study area. Identifying genotypes circulating in the study subjects would facilitate the development of effective control strategies including design of effective vaccines against *P. falciparum.*

8.2 Limitations of the study

- The numbers of alleles (bands) detected may have been underestimated due to sensitivity of PCR techniques minor fragments would not been detected on the agarose gel.
- The study had limitations like budget constraints, and inadequate some reagents and kits which may limit our findings.
- This study was not only used a large number of blood samples but also collected from one site.
- In this study different transmission seasons was not included.

9. Conclusion and recommendation

9.1 Conclusions

The results of this study showed genetic diversity and allele's distribution in MSP-1 and MSP-2 in *P. falciparum* isolated from kolla-Shelle area. A multiple infection with different strain was also observed with high frequency of MSP2 than MSP1.

The high genetic polymorphism and multiplicity of infection in Kola-shelle indicates the presence of high transmission intensity that may need consideration in malaria control program.

9.2 Recommendations

According to our findings, we would like to recommend the following points:

- This study represents a first attempt to analyze the molecular characteristic of *P. falciparum* population. However, future study needs to be designed from geographically expanded collection of *P.falciparum* field isolates from different geographic locations and different transmission intensity.
- Similar study using a large number of blood samples are required to determine the real picture of genetic profile of the country. This also helps us to evaluate the malaria control program in the country.

- All year studies covering dry and rainy seasons are needed to sense on seasonal variability.
- Microsatellite DNA sequencing is necessary to study in depth the molecular diversity of *P. falciparum* parasites infection.
- Further study association with antibody response against *MSP1* and *MSP2* is required in the study area to evaluate the impact of this polymphism on the immune response to the antigen is recommended.

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Annexes

Annex I. Patient screening form Enrollment form

1.	Patient aged 6 months and over	Yes	No
2.	Patient has severe malnutrition	Yes	No
3	Patient has mono infection with Pf	Yes	No
4	Body weight 5 kg or more	Yes	No
5	Patient with fever or history of fever in the previous 24 hours	Yes	No
6	non-pregnant or breast-feeding female	Yes	No
7	Residents living within 5-10 km radius of the health center		
8	12. Evidence of concomitant febrile illness If ―YES", indicate illness. If ―NO", leave blank. Such as, pneumonia/RTI, Measles, otitis Media , UTI , Gastroenteritis and Other:	Yes	No
9	. Evidence of severe malaria / danger signs:If ―YES" indicate criteria. If ―NO", leave blank: Unarousable coma (if after convulsion, > 30 min), Repeated convulsions (> 2 within 24 h), Recent convulsions (1-2 within 24 h),ə Altered consciousness(confusion, delerium,, coma),ə Lethargy,Unable to drink or breast feed, Vomiting everything,ə Unable to stand/sit due to weakness , Severe anaemia (Hb < 5.0 g/dL), Respiratory distress (laboured breathing at rest) and Jaundice (yellow coloring of eyes)	Yes:	No

Annex II. Written Consent Form for Study Patients

A. Written Consent Form (English Version)

Antimalarial drug called Coartem® has been used for seven years as the first line treatment for uncomplicated falciparum malaria in Ethiopia. The Federal Ministry of Health (FMOH) recommends to monitory the efficacy of Coartem® in six selected sentinel sites of which Kolla Shelle is among the selected sentinel sites. Hence, patients 6 months and above are frankly required to participate in the study. The study will be carried out in Kolla Shelle Health Center for 28 days scheduled visits in the 1st, 2nd, 3rd, 7th, 14th, 21st and 28th days of follow up and in any other days visit when you/your children feel ill health.. Coartem® will be given for 3 consecutive days, taken twice a day, the morning dose will be given in the Health Center under supervision and the evening dose will be given to adult patients or guardians of children to take it at home. Coartem® has its own side effects, so if serious adverse event on you/your child/children will happen, you/your child/children will stop the medication and get treatment with other antimalarial. This study intends to recruit agreeable patients, so you/you child/children have the right not to participate which you need to be sure there is nothing you/your child/children will loss by not participating in the study. The study will not include your/your child's/childerns' name rather is strictly confidential; the study outcome is required only to determine Coartem® efficacy in your area

which help National Malaria Control Program. For each follow up day, round trip transportation fee will be provided for enrolled patients. You can ask for more explanation if you are not clear with which will be translated in your local language.

Certificate of Content: This consent form has been readout for me in my own language and I understand the content and agree to participate (to allow my child participates) in the study.

Name of adult patient/child's parent_____ Name of witness _____

Signature_____ Signature _____

Date_____ Date _____

Name of Investigator _____

Address/es of Investigator _____

Current Address _____

Institution (s) _____Signature _____

Date _____

B. Written Consent Form (Amharic Version)

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KSÖqU Ã[ÇM:: eKJ'U እ `e− ¨ÃU MÏ− u²=I Ø—ƒ እ "Ç=d}ñ ðnÅ˜'ƒ" እ ÖÃnKG<' KSd}õ ðnÅ— J'¨< Ÿ}Ñ<–< Ÿ`e− ¨ÃU ŸMÏ− ×ƒ ¾¨v }IªeÁ'< SÉN'>ƒ ¾SssU GÃL†¨<" KSS`S` w‰ ¾UÑKÑMuƒ ƒ"i ¾ÅU Öwታ c="cÉ Ñ>²?Á© ƒ"I QSU ŸScTƒ ue}k` ¾T>ÁcŸƒK¨< U"U ‹Ó` ¾KU:: እ `e− ¨ÃU MÏ− ¾ƒ^"eþ`ƒ w`—SÅu— ¾¨v uiታ SÉN'>ƒ u'é ታÑ—L‹G<:: ¾U`U` ¨<Ö?~—S[Í‡ uUeÖ=` ÃÁ³K<:: u²=I Ø—ƒ SX}õ በ õLÔƒ w‰ c=J" uT"— ¨<U Ñ>²? ŸðKÑ< Ts[Ø Ã‹LK<:: እ `e− ¨ÃU MÏ− K28 k—ƒ ክ ትትል ይ ደ ረ ግ ል ዎ ታል፡ ፡
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64 eUU'ƒ: eK Ø—U ¯LT'U`S^—H>Åƒ }ÑMëM—M:: U¡"Áƒ dÁeðMÑ˜ ŸØ— KTs[Ø እ "ÅU‹M }[É‰KG<:: ÃI ¾eUU'ƒ pê }'xM˜'uƒ¡¡M }[ÉŠ u^c? ðnÅ˜'ƒ uØ— KS"ðM }eTU‰KG<:: K²=IU uò`TÂ ›[ÕÓ×KG<::

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Annex III : Declaration

I the undersigned, declare that this is my original work and has not been presented for a degree in this or any other university and all sources of materials used for this thesis have been acknowledged.

Name: Hussein Mohammed (BSc)

Signature _____

Place: Addis Ababa University School of Medicine Department of Medical laboratory Sciences, Ethiopia

Date of submission: _____

This thesis has been submitted with my approval as University advisor the above named student.

Name: Tedla Mendaye (BSc, MSc, PhD fellow) Signature

_____

Meseret Belayneh (BSC, MSc) Signature

_____