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Genetic diversity of merozoite surface protein-1 and 2 genes in *Plasmodium falciparum* isolates among asymptomatic population in Boset and Badewacho district, southern Ethiopia.

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This is to certify that the thesis prepared by TsegayeChekol, entitled:

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List of abbreviations

ACT………………………….. Artemisinin combination therapy

AOR …………………………..Adjust odds ratio

Bp ……………………….…Base pair

CDC …………………………Centers for disease control and prevention

CI …………………………... Confidence Intervals

DBS …………………………Dried Blood spot

DNA …………………………Deoxyribonucleic Acid

EDTA ……………………….Diamine tetra-acetic acid

GTS…………………………..Global Technical Strategy for Malaria

HMIS ………………………Health Management Information Systems

IRS………………………….....Indoor residual spraying

ITN ………………………….. Insecticide Treated Nets

MOI…………………………...Multiplicity of infection

MSP…………………………...Merozoite surface proteins

MIS …………………………...*M*anagement information system

OR ………………………….....Odds Ratio

PBS …………………………...Phosphate-buffered saline

PCR ………………………….. Polymerase chain reaction

qPCR …………………………quantitative Polymerase chain reaction

WHO………………………..World health organization

Abstract

Background: The genetic diversity of *Plasmodium falciparum* plays an important role in determining the intensity of malaria transmission.High polymorphism has been demonstrated in merozoite surface proteins 1 and 2 at different geographic locations in malaria endemic areas. This study aimto know genetic diversity of P. falciparum in the study area.

Objective: This study aimed to evaluate the Genetic diversity of *P. falciparum*merozoite surface protein-1 and 2 and genes in Boset and Badewacho district, southern Ethiopia.

Methods: A cross-sectional study wasconducted atNura Hera of upper awash agro-industry migrant farmworkers, Bosetworeda, East Shewa, southeastern and East Badewacho District, Hadiya Zone Southern Ethiopia during peak malaria transmissionfrom March to June 2020. A finger-prick blood wascollected from malaria asymptomatic individual asepticallyand screened for *plasmodium* species by preparing thick and thin blood films, and200 µl of finger-pricked dried blood spot(DBS) were used for molecular test. Then for those *P. falciparum* positive cases, genetic variation tests for merozoite surface proteins (*msp-1*) and (*msp-2*) producing was done by using Nested PCRmethods.Data were entered and analysed using SPSS version 25. The proportion of msp1 and msp2 allelic was calculated to present the distribution of different allelic families. The associations between proportions were tested using the Chi-square test. P values 0.05 were considered to indicate statistical Significance.

Results: Among738 participants 43 were had malaria, most of them were in the age group greater than 24 years; 27 (62.8%), and the mean age of the participants was 26.88 (± 15.78 SD) years. The majority of participants were male 24 (55.8%) and most of the participants 26 (60.5%) were married.Among P. falciparum *msp-1* and *msp-2* genes that were successfully amplified and analyzed, 109 different fragments were detected. Within the *msp-1* gene, a total of 54 different fragments with K1, MAD20, and RO33 had (16.3%), monoclonal infections seen. The frequencies of diclonal infections were MAD20 + K1, MAD20 + RO33 and RO33 + K1with allelic families' of20.9%, 9.3% and 4.7%, respectively. The frequencies of triclonal infections, MAD20 + K1 + RO33, were 2.3%. The multiplicity of infections for the *msp-1* genotype was 1.5. Likewise, within the msp-2 gene, a total of 55 different fragments with monoclonal infection was identified of which four and fifteen belonged to FC27 (9.3%), and 3D7 (34.9%), respectively. Diclonal infections (FC27 + 3D7) accounted for 18 (41.9%).

Conclusions: The higher the MOI in this study, the higher the prevalence of malaria in these areas and the need to strengthen control interventions.

Keywords: Plasmodium falciparum, Genetic diversity, Merozoite surface protein

1. Introduction

1.1. Background

Globally, there were an estimated 229 million cases of malariain 2019 in 87 malaria endemic countries. At the baseline of the 2015 Global Technical Strategy for Malaria (GTS) 2016-2030, 218 million cases of malaria were estimated. Malaria deaths have declined steadily over the period 2000-2019, from 736,000 in 2000 to 409,000 in 2019. An estimated 1.5 billion malaria cases and 7.6 million malaria deaths were prevented in the period 2000 -2019(1). Scaling up interventions such as widespread use of insecticide-treated nets (ITNs), indoor residual spraying (IRS), larval control, improved diagnostic testing, and artemisinin combination therapy (ACT) treatment have contributed to the decline in malaria cases around the world. An increasing number of countries, including Ethiopia, are in the process of eliminating malaria(1).

The WHO GTSset aims for malaria to reduce the incidence and mortality rates globally by at least 40%, 75% and 90% respectively, for 2020, 2025 and 2030 compared to 2015 (2). Ethiopia aims to achieve the elimination of malaria across the country by 2030 (2).

P. falciparum is one of the main malaria parasites that has been affecting millions of people around the world. The genotypic and phenotypic diversity of malaria parasites enhances their ability to counteract control measures such as therapeutic drugs. In hyper endemic areas, each individual can harbor multiple strains of malaria parasites with different genotypes, including those that confer drug resistance (3).

The genetic diversity of *P. falciparum* plays an important role in determining the intensity of malaria transmission. Several *P. falciparum* genes show extensive genetic polymorphism, as a prototype, high polymorphism has been demonstrated in merozoite surface proteins 1 and 2 (MSP-1 and MSP-2) at different geographic locations in malaria endemic areas. The *P. falciparum*merozoite gene msp-1 and msp-2 are broadly applied to investigate allelic composition and recurrence (4).

*MSP1*Polymorphic block 2 can befall within one of three different families K1, MAD20, or RO33. Different repeat sequence units characterize the K1 and MAD20 families, while The RO33 have a special order. The order in block 2 are unique to each family and are shared

between all variants. *MSP2* polymorphic block 3 can befall as one of two separate families FC27 and 3D7. Different repetitive order units are reflected in the FC27 and 3D7 families, and these repetitive regions are shared between all allelic species that are unique to each family in the series Block 3 (5).

Molecular techniques make it possible to characterize the populations of parasites present in the blood and, therefore, differentiate between recrudescence and re-infections. (6).

This study was designed to assess the genetic diversity of *P. falciparum* from Boset and Badewacho district, southern Ethiopia usingmerozoite surface protein 1 and 2.

1.2. Statement of the Problem

Malaria is a major health burden distributed in the tropical and subtropical regions of the world. It remains one of the most important causes of morbidity and mortality worldwide. Globally, it is estimated that 1.5 billion cases of malaria and 7.6 million deaths from malaria have been prevented in the period 2000-2019. Most of the cases (82%) and deaths (94%) averted occurred in the WHO African Region (1).Around 24 million children were estimated to be infected with *P. falciparum* in 2018 in sub-Saharan Africa (7).In Sub-Saharan Africa, disproportionately high share of the global malaria burden, with 90% of cases and 90% of malaria deaths, mainly in young children (8).

Malaria is a major public health problem in Ethiopia despite the relatively low prevalence of malaria compared to most other malaria-endemic African countries. Unstable malaria transmission patterns make Ethiopia prone to focal and multifocal epidemics that may have times caused catastrophic public health emergencies(9).The interaction of mountainous terrain with variable winds, seasonal rainfall, and ambient temperatures creates diverse microclimates for malaria transmission. From June 2016 to July 2017, the HMIS reported 1,530,739 confirmed malaria illnesses, including 1,059,847 *P. falciparum*(10).Ecological modification for agricultural activities such as extensive deforestation at higher altitudes in Ethiopia in the last three decades, complemented by anomalous climatic conditions that could have favored the occasional transmission of malaria. It is estimated that 52% of the population is at risk of contracting malaria(2).

Genotyping of the malaria parasite population shows several genomic polymorphisms, which constitutes a major challenge for their elimination. In molecular epidemiological studies of the malaria *msp1* and *msp2* genes, the high genetic diversity of infections at endemic malaria transmission isa considered factor, including in the intense of transmission for host immunity development. Since these genes product is considered as a candidate protein for malaria vaccine(4). Therefore, knowledge of the current genetic diversity of *Plasmodium falciparum* populations in communities is of fundamental importance to expand intervention programs. This study aimed to evaluate the genetic diversity of merozoite surface protein-1 and 2 genes in *P. falciparum* isolates among asymptomatic population in Boset and Badewacho district, southern Ethiopia.

1.3. Significance of the Study

Knowing the genetic diversity of *P. falciparum* is very important to inform policy makers about intervention strategies. Indicating the type of allelic family of *P. falciparum* most predominant is essential to design management strategies. To date, there has been a limited assessment of the genetic diversity of *P. falciparum* in Ethiopia. Study results can also help national and local health centers and interested health offices to understand the burden of malaria and genetic diversity in the study area and to plan a well-organized malaria prevention and control program. In addition, the study will be used as recent information for those who need to do more research in the area.

2. Literature review

In the study that was done in Indonesia, analysis of clinical signs of fever and chills was a very common indication. Msp1 gene analysis showed the presence of allele subtype 42, 34, and 1 in MAD20, K1, and RO33 subjects, respectively. The mixed K1 + MAD20, K1 + RO33, and MAD20+RO33 in 5, 4, and 4 subjects, respectively. Whereas msp2 gene analysis revealed 37 and 34 subjects carried 3D7 and FC27 allelic subfamily, respectively, and 19 subjects carrying FC27 + 3D7 mixed. The MOI analysis showed that the msp1 and msp2 alleles with an average MOI of 2.69 and 2.27, respectively. The combination of K1 + RO33 msp1 was correlated with critical malaria (OR: 28.50; 95%CI: 1.59–1532.30) (11).

According to a study conducted in Myanmar, the allelic frequencies of the three genes were very different between the three areas. MAD20 and 3D7 were the most widespread alleles in three areas. Overall, 73.91% of samples had multiple infections, with an average MOI of 1.94. Interestingly, the MOI level is in line with the growing demand for Myawaddy, Kyauktaw, and Shwekyin orders, which is in line with the increasing frequency of Msp1 RO33 and Msp2 FC27 200-250 bp alleles (12).

The study in central India, the malaria positivity was found 26% in 2005, which rose to 29% in 2009 and *P. falciparum* prevalence was also increased from 72% in 2005 to 81% in 2009. The overall allelic prevalence was higher in K1 (51%) followed by MAD20 (28%) and RO33 (21%) in 2005 while in 2009, RO33 was highest (40%) followed by K1 (36%) and MAD20 (24%) (13).

The study done in *P. falciparum* clinical isolate from Lao PDR the overall prevalence of K1, MAD20 and RO33 allelic types in *P. falciparum* isolates from Lao PDR were 66.95%, 46.52% and 31.30%, respectively, of samples under study. Single infections with K1, MAD20 and RO33 allelic types were 27.83%, 11.74% and 5.22%, respectively; the remainders were multiple clonal infections. Neither parasite density nor age was related to MOI. Sequence analysis revealed that there were 11 different types of K1, eight different types of MAD20, and 7 different types of RO33. Most of them were regional specific, except type 1 of each allelic type was common found in 3 regions under study (14).

In eastern Malaysia, the MSP-1 and MSP-2 alleles were predominantly family types K1 and FC27, respectively. The MOI were 1.65 and 1.20 in the MSP-1 and MSP-2, respectively. In Kota

Marudu, the MSP-1 and MSP-2 alleles predominantly comprised the MAD20 and 3D7 family types. The MOI MSP-1 and MSP-2 were 1.05 (15).

In the study of Western Cambodia, significant differences between msp2 alleles and well-preserved msp1 were recognized. Initially, polyclinical infection were 31% to one or more genes. Patients with recurrent malaria were high more likely to develop a polyclonal infection than non-recurrent patients (p = 0.004) (16).

A study of north-central Nigeria identified a total of 26 different msp-2 alleles from 215 fragments. Most of the isolates were polyclonal infections consisting of 2–6 clones and were most common in the FC27 allelic family (p = 0.036). The study found that in the population as a whole, 2.31 per household were hadmultiplicity of infection, with the majority MOI ranging from 1.0 to 4.5. Family distribution patterns of msp-2 allele types are divided into two categories: families with both msp-2 allele types FC27 and 3D7; Families with only one msp-2 allele type FC27 or 3D7 were present. Although most households have both types of msp-2 allele, they are evenly distributed among children, and in a few families, all children have only one type of msp-2 allele (17).

In a study of Equatorial Guine, three msp-1 alleles and two msp-2 alleles were analyzed in all samples. In msp1, the MAD20, K1, and RO33 allelic families were 96.69%, 96.07%, and 70.78%, respectively. In msp-2, the FC27 and 3D7 allelic family was found to be 97.69% and 72.25%. Twenty-six different alleles in msp1 were shown with 9, 9, and 8 alleles for K1, MAD20, and RO33, respectively. In msp-2, 25 individual alleles were found in 20 and 5 alleles for 3D7 and FC27, respectively. The overall score MOI for msp-1 and msp-2 was 5.51 with 3.5 and 2.01 respectively. There was a meaningful increase in overall MOI Standing on the age group of patients (P = 0.026)(18).

In the study in Cameroon, MSP-1, 16 genotypes were identified, including K1, MAD20, and RO33. The difference in this study was the RO33 monomorphic pattern described between the msp-1 allelic types. Again, this study 27 different msp-2 genotypes identified, including the15 and 12 for 3D7 and FC27 allelic families. Analysis of MSP-1 and MSP2 peptides showed that the K1 polymorphic alignment region had significant similarities in MSP1 and MSP2 clade following MAD20 with 93% to 100% homology. Therefore, vaccines developed with K1 and

Pfmsp1 allelic version MAD20 may be protective for African children, but these findings require more genetic and pathological testing, similar to other areas in Africa (19).

The study in a malaria endemic area of Burkina Faso, the distribution of the msp1 and msp2 allelic families was not different according to haemoglobin type (p=0.70 and 0.90 respectively) and G6PD type (p=0.89 and 0.82 respectively). The analysis of the mean multiplicity of *P. falciparum* infection (MOI) based on haemoglobin variants showed msp1 with high values 2.96 and 3.12 for Normal haemoglobin and abnormalhaemoglobin respectively. However, according to the G6PD type, there were no differences of MOIs between normal G6PD and deficient G6PD carriers. The study showed the *P. falciparum* genetic diversity was not affected by human genetic factors based on the analysis of *msp1, msp2*(20).

The study in Mali,of 156 qPCR-positive samples, complete genotyping of 112 samples was achieved. The parasite populations displayed high genetic diversity (mean He = 0.77), which was consistent with a high level of malaria transmission in Mali. Genetic differentiation was low (FST < 0.02), even between sites located approximately 900 km apart, thereby illustrating marked gene flux amongst parasite populations. The lack of linkage disequilibrium further revealed an absence of local clonal expansion, which was corroborated by the genotype relationship results. In contrast to the stable genetic diversity level observed throughout the country, mean multiplicity of infection increased from north to south (from 1.4 to 2.06) and paralleled malaria transmission levels observed locally (21).

In a study conducted in Lagos Nigeria, all the *msp-1* and *msp-2* were all observed. In msp-1 K1, MAD20, and RO33 was (60/100), (50/100) and (45/100) frequency, respectively. In *msp-2*, FC27 and 3D7 frequency (62/100) and (55/100), respectively. Allelic families were isolated and / or united with other families. However, no R033 / MAD20 combination was observed. The incidence of infection with *msp-1* was higher than that of Ikoroduand Lekki1.50 and 1.39 wereas in msp-2 Ikoroduand Lekki 1.14 and 1.76. However, there was no remarkable difference in the average MOI between the two study fields (P = 0.427)(22).

Polyclonal infections were more common in patients with *MSP-1* allelic families than in monoclonal infections *MSP-2* allelic families. For MSP-1, and *MSP-2* the Multiplicity of infection rates were 1.7 and 1.8, respectively. (4).

In Central Sudan, 11 were identified as *MSP1* block 2 in the White Nile State of Costa Rica and 16 others were identified as MSP2 block 3. In MSP1, RO33 carried alone or in combination with the MAD20 and K1 variants, the FC27 family was found to be the most widespread in MSP2. 62% of the isolates had multiple genotypes and the total MOI was 1.93 (CI 95% 1.662.20) (23).
A study in the Republic of the Congo identified 468 distinct fragments, *msp-1*and msp-*2, 15 and 20* genotypes, respectively. For the *msp-1* gene, the K1 family wasthe widespread isolated or in combination with the RO33 and MAD20 subfamily, and the 3D7 family was major widespread in the *msp-2* gene. Overall, the average MOI was 2.2. 83% of the 125 samples contained more than oneparasitegenotype. There were no remarkable differences in the severity of the disease, either in sex or in patients' age. (24).
Studies in Ghana's high and low malaria transmission cohort, as well as the prevalence of asymptomatic *P. falciparum* carriers and the prevalence of infectious pathogens in Obom were significantly higher than in Asutsuaregenotyping was 100% and 65% clonal, respectively, from Asutsuare and Obomsamples, but decreased by 50% and 5%, respectively, according to MS analysis in msp-2 genotyping. The parasites of Obom and Asutsuare were very different, and the parasites of Obom were more diverse thanAsutsuare(25).
The total MOI in Sudan was uncomplicated andsevere malaria 2.25, 2.30, and 2.15, respectively. There were no remarkable differences between uncomplicated and severe malaria patients in the MOI with respect to *MSP1, MSP2*, and general MOI. The high MSP1 allelic families were MAD20 and RO33 with uncomplicated malaria and severe malaria, respectively. The distribution of MSP2 FC27 and IC1 / 3D7 allelic families was approximately similar in disease severity. 111 *P. falciparum* 81% containing several genotypes; 71/90 in uncomplicated malaria and 40/50 in severe malaria patients. *MSP1 and MSP2* allelic families were not associated with severe malaria. There were no remarkable differences in MOI between different age groups(26).
A study of Sudan's two geographical zones found a total of 241 samples (88.9%) positive for *P falciparum*. There were 14, 15, 13 and 12 different *MSP2* alleles in Khartoum, Gujarat, the Nile River and the Red Sea. The 3D7 allelic family was more widespread in Khartoum, the Gezira, the Nile, and the Red Sea than the FC27 allelic family. Multiple infections were reported in 25.8% of patients, with an average MOI of 1.45. MOIs were higher in the age group over 40,

with an average of 2 and 1.68 in Khartoum and Gezira states, but in the Nile and Red Sea states, MOIs were higher in the under-18 age group, averaging 1.37 and 1.33, respectively (27).

Ina study in Western Kenya, 10 polymorphic microsatellite markers were evaluated. On average, 79.69% of the exclusions analyzed in this study were polyclonal infections. The highest average MOI is 3.39 and 0.81. The samples analyzed included a large number of polyclonal infections that led to the spread of a wide range of genetic parasites (28).

In northwestern Ethiopia, 95 (80.5%) and23 (19.5%) of uncomplicated infections and were severely ill respectively. In *msp-1*, the K1 allelic family was widespread uncomplicatedand severe cases in 42 (44.2%) and 12 (52.2%), respectively. In *msp-2*, FC27 and IC / 3D7 was found in 55 (57.9%) and 14 (60.9%) uncomplicated infections and in severe infections, respectively. Of the 118 isolates, 76 (64.4%) contained multiple genotypes; 56 (58.9%) and19 (82.6%) with uncomplicated infections and with severe infections, respectively. TotalMOI were 2.2 (95% CI 1.98-2.42) at *msp-1*with 1.4 and msp-2 at 1.7. Uncomplicated infections 3.0 and severe 2.0, p = 0.001) were significantly higher. There was no remarkable difference in disease MOI among age groups (p = 0.104)(29).

Northwestern Ethiopia, *msp-2* for *msp-1* for 12 and 22, respectivelyfrom 34 *msp* alleles found in a total study. In the *msp-1 and msp-2* was found for 97.8% and 82.2%. In *msp-1*, MAD20 was identified as a family member with 47.7% following RO33 and K1. For *msp-2*, the distribution of FC27 were 77% and IC / 3D7 were 76%. The *msp-1*, and *msp-2*heterozygosity index was 0.82, and 0.62, respectively. There was no remarkable relation between MOI and age (30).

A study in southwestern Ethiopia identified a difference in *msp-1* and *msp-2* 67% and 44% were identified, respectively. K1 was the major *msp-1* allelic family in 33.9% of the samples followed by RO33 and MAD20. The *msp-2* allelic family 3D7 / IC1 and FC27 showed distribution 21.5% and 10.3%, respectively. 59% of the isolates had multiple genotypes and the overall infection rate was 1.8.There were no remarkable differences in the multiplicity of infection (P> 0.05), regardless of age(31).

The msp2 alleles, overall 22 alleles for 3D7 / IC and FC27 were 11 and 11 allelic family, respectively, were studied in northwestern Ethiopia in the province of Pawe. The preferred frequency allelic family of msp2 3D7 / IC was 51% higher compared to FC27 49%. Most 76%

excluded multiple infections, with a total infection rate of 2.8. The heterozygosity index msp2 was 0.66. There are no remarkable differences in in the multiplicity of infection by age (32).

A total of 29 alleles for msp-1 and msp-2 were found in the Chewaka district of Ethiopia. In msp-1, K1 was the main allelic family in 47.7% of the samples followed by MAD20 and RO33. For msp-2, the frequency of FC27 was 77% and IC / 3D7 was 76%. 80% of the isolates had multiple genotypes and the overall MOI was 3.2 There was no remarkable relationship between multiplicity of infection and age (33)

3. Objective

3.1. General Objective

To evaluate genetic diversity of merozoite surface protein-1 and 2 genes in *Plasmodium falciparum* isolates among asymptomatic population in Boset and Badewacho district, southern Ethiopia.

3.2. Specific Objectives

- To characterize genetic diversity of *Plasmodium falciparum* msp-1 and msp-2in Boset and Badewacho district, southern Ethiopia.
- To determine the prevalence of *msp-1* and *msp-2* allelic families in Boset and Badewacho district, southern Ethiopia.
- To evaluate multiplicity of infectioninBoset and Badewacho district, southern Ethiopia.

4. Materials and Methods

4. Methods and Materials

4.1. Study Area and Period

This study was carried out in in stored sample collected fromNura Hera of upper awash agro-industry migrant farmworkers, Boset District, East Shewa, southeastern and East Badewacho District, Hadiya Zone Southern Ethiopia. The Boset district is bordered to the south by the Arsi area, to the west by the Awash River which separates it from Adama, to the north by the Amhara region and to the east by Fentale. The altitude of the district varies from 1100 to 2700 m above sea level and receives an annual average of rainfall that varies between 700 and 800 mmHg, the intensity and variability being high in the district. The district is characterized by a hot and dry climate with an average annual temperature that varies between 25 and 30 ° C for the tropical (Kolla) and between 15 and 20 ° C for the subtropical (woinadega). Precipitation is weakly bimodal with spring (a small rainy season) during the months of April and May, while summer (a long rainy season) during the months of July to September. The data obtained from the population projection by the Central Statistical Agency (CSA) indicated that the total population of the Boset district for the year 2017 was projected at 189,795 of which 42,793 (22.5%) are urban population and 147,002 (77.5%) are rural population (88). In terms of the drainage system, the district falls into the Awash River basin, with no other major streams and lakes. It is an agricultural area where extensive agriculture is carried out in Ethiopia through the irrigation of the Awash River. This area is known to be malarial with intense transmission patterns.

East Badawacho has 225 km away from the capital city, Addis Ababa. East Badawacho is bordered on the South by the Wolayita Zone, on the West by MirabBadawacho, on the North by the KembataTembaro Zone, on the North East by the Alaba special woreda, and on the East by the Bilate River which separates it from the Oromia Region. The altitude of the district ranges from 1501-2500 meters above sea level and receives a mean annual rainfall of 801-1400 mm Hg. The average annual temperature ranges from 17.6-22.5 To in Centigrade. Based on the 2007 Census conducted by the CSA, this woreda has a total population of 142,823. Agriculture is the principal source of livelihood for the rural population.

4.2. Study Design

A cross-sectional study was conducted from March 2020 to June 2021, inBoset and Badewacho district, southern Ethiopia.

4.3. Population

4.3.1. Source population

The source population was all migrant farmworkers living in Boset and Badewacho district, southern Ethiopia.

4.3.2. Study Population

All asymptomatic migrant farmworkers who lived in Boset and Badewacho district, the data was collected March to June 2020.

4.3.3. Study Participants

All asymptomatic migrant farmworkers lived in selected householdsin Boset and Badewacho district who fulfill the inclusion criteria.

4.3.4. Eligibility

4.3.4.1. Inclusion criteria

Family members, living in the selected households,lived at least for the last six months in the study area and found during data collection whowith no disease symptoms/signs of malaria in the last four (4) days and axillary temperature 37 ° C in Boset and Badewacho district, southern Ethiopia.

4.3.4.2. Exclusion criteria

Individuals who tookanti-malarial therapy at the time of data collection or who had been treated with anti-malarial drugs within the past month before enrolment.

4.4. Sample size determination and sampling technique

In East BadewachoDistric the sample size for the first objective was determined by using a formula for a single population proportion

$$n = \frac{Z_{\alpha/2}^2 p\,(1-p)}{d^2}$$

Where n = the sample size, Z /2 = 1.96 at 95% confidence interval (CI), d= margin of error, p = expected malaria prevalence rate in which was assumed 50%, because previously, there is no study was conducted in area(nearer to study area) and in the country. Margin of error (d) at 5%, design effect =1.2 and non-respondent rate = 5%.

$$n = \frac{1.96 \times 1.96 \times 0.5\,(1-0.5)}{0.0025} = \mathbf{384}$$

Design effect = 1.2 n = 384x1.2 = **461**

Therefore, total sample size =461+ (461x5%) =484 individuals

In the area Bosetworeda, the sample size was determined using a single population proportion formula using the following assumptions; p = 18.4% prevalence of asymptomatic malaria among migrant workers from the (34), the prevalence of the previous study found from literature review=18.4%, 0.184

d = Margin of error at (5%) (0.05)

n = ((1.96)2x 0.184(1-0.184))/(0.05)2

n = 231

10% non-response rate=23, so the total sample size (n) will be n=231 + 23 = 254

The total sample size is 484+254= 738

Where: from 738asymptomatic population,microscopic confirmed 43 *P. falciparum*stored samplewas total sample size.

4.5. Sampling Techniques

Boset and Badewacho district were selected using conventional sampling techniques. Each study participant was selected using a systematic random convenient sampling technique. If there were more than one person per household, one was randomly select.

4.6. Blood sample collection and processing

Socio-demographic data like age, sex, and marital status was collected by trained health professionals using a pre-tested structured questionnaire the questionnaire had adapted from other similar studies and prepared in English. It had back-translated into local language. Finally, it was translated back into English to check for consistency.

Approximately three to four drops of finger-pricked blood were spotted on 3 mm Whatman filter paper, dried, and placed in airtight plastic bags with desiccant and stored at –20°C in the parasitology research laboratory at Aklilu Lemma institute of Pathobiology, Addis Ababa University.

4.7 Genomic DNA extraction

Genomic DNA was extracted from dried blood spots using the Chelex-saponin method and the final extracted genomic DNA samples stored at -20 oC until used for PCR amplification as described previously(35)

4.8 Allelic typing of *MSP1* and *MSP2*

Allelic typing were performed using primer specific for the polymorphic regions of *P.falciparummsp-1* (block 2) and *msp-2* (block 3). The two round PCR amplifications were performed as described previously (36). In the primary PCR reaction, primers span the whole genetic locus of *msp-1* (block 2) and *msp-2* (block 3), while secondary /nested PCR reaction target family specific allele of *msp-1* (K1, MAD20 and RO33) and *msp-2* (FC27 and 3D7) (Table 1). Both the primary and nested PCR reactions were performed in a final volume of 20 µl containing 0.25 µM of each primer and 1 unit of 5X hot fire pol master mix. In the primary PCR reaction, 4 µl of DNA template was used, while in the secondary PCR reaction, 2 µl of primary PCR products were used. The cycling conditions for the Primary PCR were initial denaturation at 95 ° C for 3 min, followed by 35 cycles of denaturation at 95 ° C for 60 sec.; annealed at 58 ° C for 1 min; extension at 72 ° C for 90 sec and a final extension at 72 ° C for 60 seconds and

holding at 10 ° C. The cycling conditions for secondary PCR are the same as primary PCR except the number of cycle, 30 cycles for nested PCR. The PCR products were stored at 4 ° C until analysis.

Five microliters (6 µl) of the amplified products were electrophoresed using 2% agarose gels made in Tris-borate-EDTA for *P. falciparum*. The PCR products was then stained with ethidium bromide for visual detection by ultraviolet transilluminator light (37)(15). The PCR were carried out at Addis Ababa University, Aklilu Lemma Institute of Pathobiology.

Table 1: Sequences of the primers for *msp-1* and *msp-2* genes in *P. falciparum* isolates

Locus	PCR Round	Primer	Primer Sequences	Reference
Msp-1 (block 2)	**Primary PCR**	msp1-F1	5'-CTA GAA GCT TTA GAA GAT GCA GTA TTG-3'	
		msp1-R1	5'-CTT AAA TAG TAT TCT AAT TCA AGT GGA-3'	
	Secondary PCR	K1-F	5'-AAT GAA GAAGAA ATT ACT CA AAA GGT-3'	
		K1-R	5'-GCT TGC ATC AGC TGG AGG GCT TGC ACC-3'	
		MAD20-F	5'-AAA TGA AGG AAC AAG TGG AAC AGC TGT-3'	
		MAD20-R	5'-ATC TGA AGG ATT TGT ACG TCT TGA ATT-3'	
		R033-F	5'-TAA AGG ATG GAG CAA ATA CTC AAG TTG-3'	
		R033-R	5'-CAT CTG AAG GAT TTG CAG CAC CTG GAG-3'	
Msp-2 (block 3)	**Primary PCR**	msp2-F1	5'-ATG AAG GCA ACT AAA ACA TTG TCT ATT-3'	
		msp2-R1	5'-CTT TGT TAC CAT CGG TAC ATT CTT-3'	
	Secondary PCR	3D7-F	5'-GCA GAA AGT AAG CCT TCT ACT GGT GCT-3'	
		3D7-R	5'-GAT TTG TTT CGG CAT TAT TAT-GA-3'	
		FC27-F	5'-GCA AAT GAA GGT TCT AAT ACT AAT AG-3'	
		FC27-R	5'-GCT TTG GGT CCT TCT TCA	

4.9 Ethical considerations

Ethical clearance was obtained from School of medical Laboratory Sciences, college of health sciences, University of Addis Ababa, ethical review committee. Stored sample are taken from parasitology research laboratory at Aklilu Lemma institute of Pathobiology, Addis Ababa University.

4.10 Operational Definitions

Plasmodium falciparum**:** is the etiological agent of malaria, the leading cause of death due to a vector-borne infectious disease

Genetic diversity:the variation in the genetic composition among malaria, a species, an assemblage, or a community. Diversity on a genetic level is a reflection of the similarities and differences in the genes (segments of DNA on chromosomes).

Merozoite surface protein: are embedded on the surface of the merozoite and the inner layer proteins found on the surface of a merozoite, an early life cycle stage of a protozoanmsp-1 and msp-2.

msp-1:merozoite surface protein 1 of *P. falciparum*

msp-2:merozoite surface protein 2 of *P. falciparum*

5. Work flow

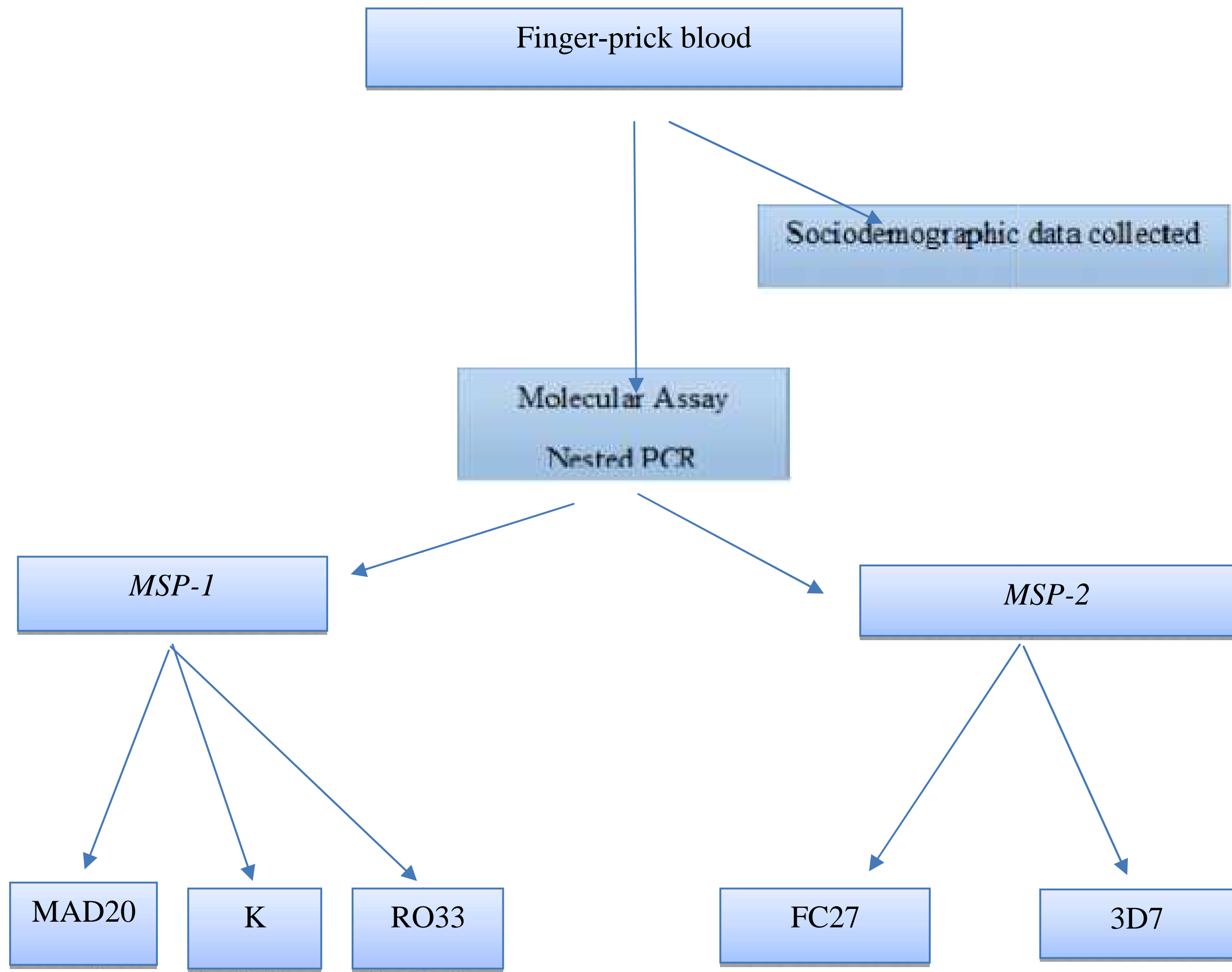


Fig. 1. Showing the work flow of the study

6. Results

6.1. Socio-demographic Characteristics

A total of 43 sample were included in this study. Most of the participants were male, 24 (55.8%). The mean age of the participants was 26.88 (± 15.78 SD) years, which ranged from 2 to 80 years. Most of the participants were in the age group greater than 24 years 27 (62.8%). Besides, most of the participants were married26 (60.5%), and 1 (2.3%) was divorced (Table 2).

Table 2: Socio-demographic characteristics of the participants, South-Eastern and South, Ethiopia,April to June 2020.

Variables		Number (%)
Sex	Male	24(55.8%)
	Female	19(44.2%)
Age	0-5	2(4.7%)
	6-14	8(18.6%)
	15-24	6(14.0%)
	>24	27(62.8%)
Marital status	Single	16(37.2%)
	Married	26(60.5%)
	Divorced	1(2.3%)

6.2 Genetic diversity and allele frequency of *P. falciparummsp-1* and *msp-2*

Allelic polymorphism of *msp-1* and *msp-2* genes of the parasite DNA of the 43 isolates of *P. falciparum* was analyzed. A total 109 different fragments of *msp-1* and *msp-2* were detected. Within the *msp-1* gene, a total of 54 different fragments with seven belonged to K1 (16.3%), seven belonged to MAD20 families (16.3%), and seven belonged to RO33 (16.3%). %), respectively, were monoclonal infections. The frequencies of diclonal infections were 20.9%, 9.3% and 4.7% were allelic families of MAD20 + K1, MAD20 + RO33, and RO33 + K1, respectively. The frequency of infection by triclonal MAD20 + K1 + RO33 was 2.3%. The MOI

value for the*msp-1* genotype was 1.5. Within the *msp-2* gene, a total of 55 different fragments with four belonged to FC27 (9.3%), and fifteen (34.9%) belonged to 3D7 monoclonal infection families had seen. The eighteen (41.9%) diclonal infection frequencies were FC27 + 3D7 allelic families (Table 2). The MOI value for the *msp-1* genotype was 1.5 (table 3).

Table 3Genetic diversity and allele frequency of *Plasmodium falciparum* msp1 and msp2 from Southeastern and Southern, Ethiopia,April to June 2020.

	Family types	Number (%)	MOI
MSP1	MAD20	7(16.3%)	1.5
	K1	7(16.3%)	
	RO33	7(16.3%)	
	MAD20+K1	9(20.9%)	
	MAD20+RO33	4(9.3%)	
	RO33+K1	2(4.7%)	
	MAD20+K1+RO33	1(2.3%)	
	Total	43	
MSP2	FC27	4(9.3%)	1.5
	3D7	15(34.9%)	
	FC27+3D7	18(41.9%)	
	Total	43	

6.3 Status of Multiclonal infections

Of the *msp-1* and *msp-2* successfully genotyped for all three allelic families, only 48.9% samples were mono- allelic and 44.2% sample were mono-allelic at each of the two genes, respectively (fig.2).

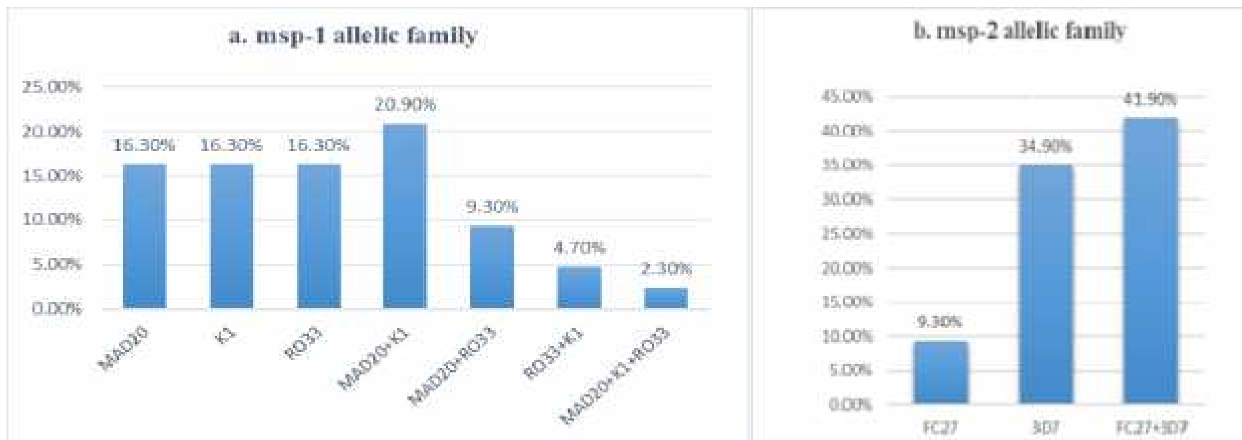


Fig. 2a. *msp-1* allelic families, b. *msp-2* allelic families

The distribution of specific allelic families of both *msp-1* and *msp-2* with respect to age group of the study participants were not statistically significant (p > 0.05, two-proportion test). MOI was different among age groups, but the highest MOI 1.48 and 1.52 in older age group (>24 years) in *msp1*, and *msp-2* respectively. Likewise, multiple clonal infections, and the each allelic type was not significantly different (p > 0.05, two-proportion test) in *msp-1* and *msp-2*, is shown in Table 4.

Table 4. Allelic types of *msp-1 andmsp-2* distribution among age groups

***msp-1* and *msp-2* allelic type**	**Age group (years)**				**Total No. (%)**
	0-5 No. (%)	6-14 No. (%)	15-24 No. (%)	>24 No. (%)	
MAD20	0	1(2.7%)	1(2.7%)	5(13.5%)	7(18.9%)
K1	0	1(2.7%)	1(2.7%)	5(13.5%)	7(18.9%)
RO33	0	2(5.4%)	1(2.7%)	4(10.8%)	7(18.9%)
MAD20+K1	1(2.7%)	2(5.4%)	1(2.7%)	5(13.5%)	9(24.3%)
MAD20+RO33	0	1(2.7%)	0	3(8.1%)	4(10.8%)
K1+RO33	0	0	0	2(5.4%)	2(5.4%)
MAD20+K1+RO33	0	0	0	1(2.7%)	1(2.7%)
Multi-clonal isolates	1(2.7%)	3(8.1%)	1(2.7%)	11(29.7%)	16(43.2%)
MOI	1.0	1.43	1.25	1.48	1.46
Total	1(2.7%)	7(18.9%)	4(10.8%)	25(67.6%)	37(100%)

FC27	0	0	0	4(10.8%)	4(10.8%)
3D7	2(5.4%)	3(8.1%)	3(8.1%)	7(18.9%)	15(40.5%)
FC27+3D7	0	3(8.1%)	3(8.1%)	12(32.4%)	18(48.6%)
Multi-clonal isolates	0	3(8.1%)	3(8.1%)	12(32.4%)	18(48.6%)
MOI	1.0	1.5	1.5	1.52	1.49
Total	2(5.4%)	6(16.2%)	6(16.2%)	23(62.2)	37(100%)

The distribution of specific allelic families of both *msp-1* and msp-2 with respect to sex group of the study participants were not statistically significant ($p > 0.05$, two-proportion test). MOI was different among sex groups, MOI 1.47 and 1.44 of male and female in *msp-1* respectively and 1.45 and 1.53 of male and female in *msp-2* respectively, is shown in Table 5.

Table 5. Allelic types of *msp-1* and *msp-2*distribution among sex groups

	Sex		**Total No. (%)**	**P-value**
	Male No. (%)	Female No. (%)		
MAD20	3(5.56%)	4(7.4%)	7(12.96%)	0.45
K1	4(7.40%)	3(5.56%)	7(12.96%)	
RO33	3(5.56%)	4(7.40%)	7(12.96%)	
MAD20+K1	8(14.81%)	10(18.52%)	18(33.33%)	
MAD20+RO33	6(11.11%)	2(3.70%)	8(14.81%)	
K1+RO33	4(7.40%)	0	4(7.40%)	
MAD20+K1+RO33	0	3(5.56%)	3(5.56%)	
MOI	1.47	1.44		
Total	28(51.85%)	26(48.14%)	54(100%)	
FC27	11(20.37%)	4(7.14%)	15(26.79%)	0.2
3D7	1(1.85%)	3(5.36%)	4(7.14%)	
FC27+3D7	20(35.71%)	16(28.57%)	36(64.29%)	
MOI	1.45	1.53		
Total	33(58.93%)	23(41.07%)	56(100%)	

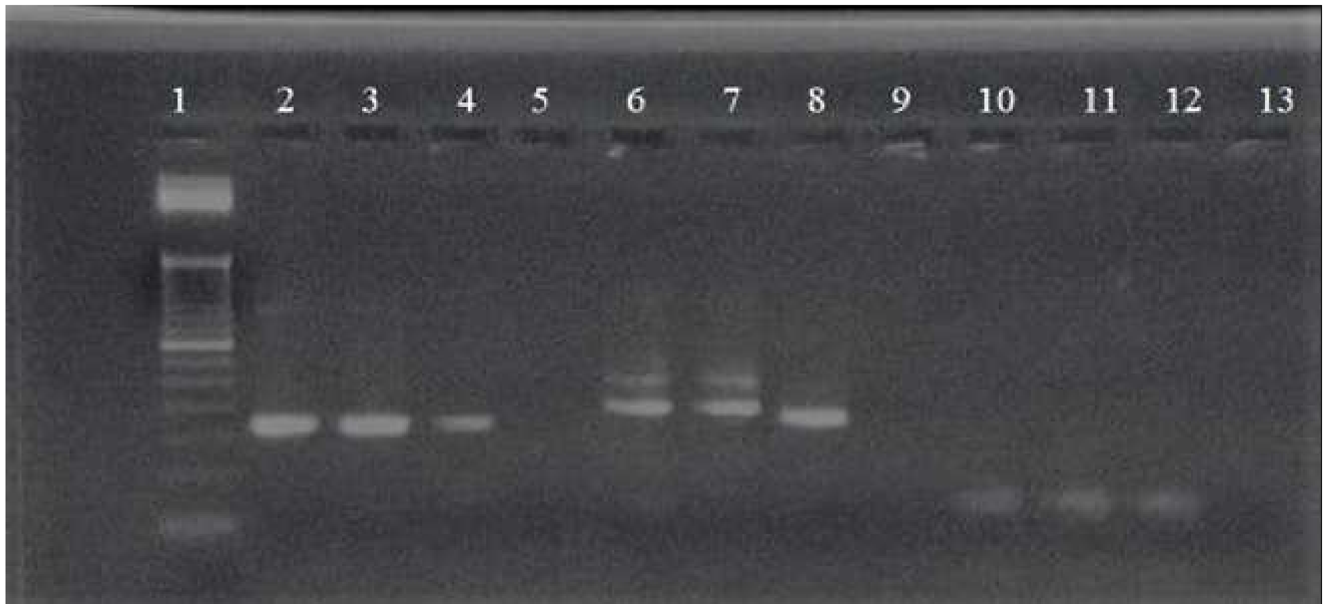


Fig 3. Alleles of the *msp-1* gene showing; Lane 1: 50 bp ladder Lane 5, 9, and 13: negative control and Lane: 2, 3, 4MAD20Lane: 6, 7, 8 K1Lane: 10, 11, 12RO33are positive for allelic families

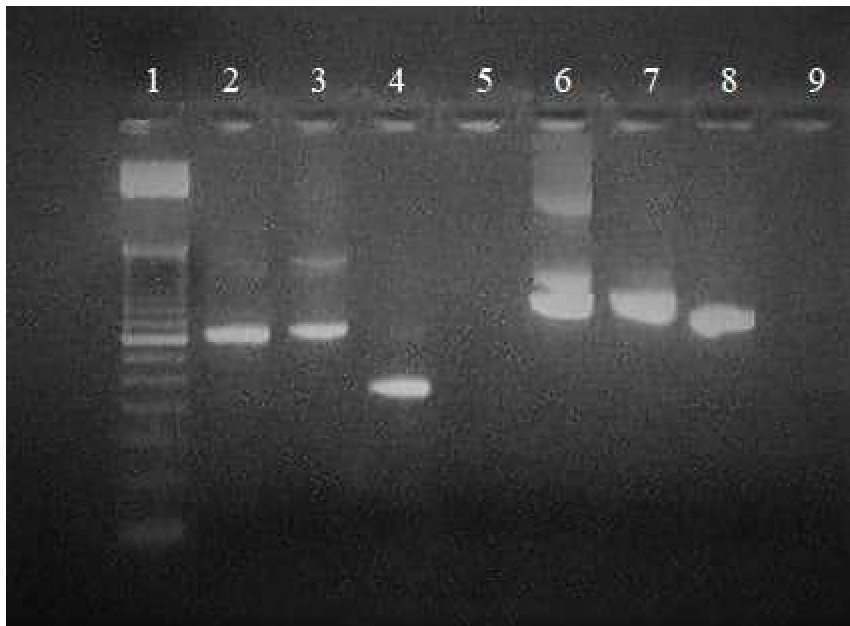


Fig 4. Alleles of the *msp-2* gene showing both FC27 lane 2, 3, 4 and 3D7 lane 6, 7, 8positive for allelic families, Lane 1: 50 bp ladder, and Lane 5 and 9 negative control

7. Discussion

The purpose of this study was to determine the molecular properties of polymorphic markers in the origin of *P falciparum*. In this study, the field of *P. falciparum* showed significant genetic polymorphism in families of *msp-1* and *msp-2* genes in southeastern and southern Ethiopia. Moreover, a significant contribution to multiclonal infection was identified. A special feature of this study is that the MSP-1 allelic families MAD20, K1 and RO33 were identified with equal frequency unlike other studies(4)(11)(24)(31)(20). MAD20 and K1 mixed infection Polymorphic and the most advanced msp-1 allelic families MAD20 and K1 have been found to agree with studies that have been shown to be the most important allelic family for the MSP-1 locus (11)(24)(33). However, the results of this study do not confirm other studies that have identified MAD20 and RO33 as the main allelic family of msp-1 block. (26).

The *msp-2* allelic families 3D7 were almost the most predominant allele type of allelic families in agreement with previous studies where 3D7 was found to be the predominant allelic family for msp-2 locus (20)(31)(38)(16). In contrast, previous reports showed a significant predominance of FC27 over the 3D7 allelic family (33). Current studies show that the msp-2 gene is much more polymorphic compared to the *msp-1* gene. This observation was remarkable different in the low transmission settings recorded for *msp-1* compared to the *msp-2*gene (26). Therefore, msp-1 and

msp-2 differed in the frequency of recurrence and recurrence of the disease with the low frequency of individual transmissions observed in this study. Therefore, this study, based on msp-1 and msp-2, emphasizes the importance of *P. falciparum* genotyping in anti-malarial experiments, emphasizing the importance of recurrence of the infection and the effectiveness of msp-1 and msp2. Malaria control and malaria control strategies in Ethiopia and elsewhere.

Although there are many control strategies in health facilities and in the community, the high MOI in this study is widely used in southern Ethiopia. This finding is consistent with previous findings with an increase in MOI compared to other studies(31)(22) greater than(15)(25), and lower than some others (38)(19) studies. The MOI in the older group (> 24 years) was 1.48 and 1.52 higher in msp-1, and msp-2. This agrees with the previous finding (27) and lower than the other finding (18). MOI 1.47 and 1.44 male and female in msp-1, respectively 1.45 and 1.53 male and female in msp-2, but no results were compared.

Therefore, regular molecular epidemiological surveys need to be performed in order to monitor the genetic diversity of P. falciparum populations in different regions of Ethiopia. This study showed an increase in the MOI according to the gender of patients which is consistent with previous studies showing significantly high MOI in patients with moderate to high transmission (31). In the present study, most of the positive samples were from aged >24 years. Thus, determining the MOI in endemic areas is very important since it can be used to predict clinical outcome and target population with higher attention.

8. Strengths and Limitations of the Study

The present study has vigorously genotyped the *P. falciparum* isolates on 2 antigenic (*msp-1* and *msp-2*) markers with efforts to obtain a conclusive genetic diversity dataset for the areas. The limitation of the present study was the small sample size

9. Conclusion

The present study shows that field isolates of Boset and Badewacho district, southern Ethiopia were found to be mainly polyclonal with high MOI and highly diverse in respect to both msp-1 (block 2) and msp-2 (central repeat region, block 3. This study lays emphasis on the use of both

msp-1 and msp-2 genes in monitoring the trend of malaria epidemiology and the use of MOI as an important indicator in the evaluation of malaria control interventions. The high MOI observed in this study is an indication that malaria transmission remains high in area calls for intensifying control interventions. .

10. Recommendations

This study show high MOI important indicator in the evaluation of malaria control interventions. The high MOI observed in this study is an indication that malaria transmission remains high in area calls for intensifying control interventions. The respective governmental and on governmental stakeholders in the areas need to collaborate more to rig the desiredmalaria control and prevention actions.

11. References

1. 2020 W. World Malaria Report 2020 [Internet]. Vol. 73, Who. 1997. 1–4 p. Available from: https://www.who.int/teams/global-malaria-programme/reports/world-malaria-report-2020
2. Ababa A. Ethiopia malaria elimination strategic plan: 2021-2025. 2021;(August 2020):2021–5.
3. Robinson T, Campino SG, Auburn S, Assefa SA, Polley SD, Manske M, et al. Drug-Resistant Genotypes and Multi-Clonality in Plasmodium falciparum Analysed by Direct Genome Sequencing from Peripheral Blood of Malaria Patients. 2011;6(8).
4. Centre H, State K, Kolawole OM, Mokuolu OA, Olukosi YA, Oloyede TO. Population genomics diversity of Plasmodium falciparum in malaria patients. 2016;16(3):704–11.
5. Snounou G. Genotyping of Plasmodium spp. Nested PCR. 2014;(November).
6. Bakary M, Keundjian A, Mbomat B, Makita JR, Migot-nabias F, Cot M, et al. Combination of Drug Level Measurement and Parasite Genotyping Data for Improved Assessment of Amodiaquine and Sulfadoxine-Pyrimethamine Efficacies in Treating Plasmodium falciparum Malaria in Gabonese Children. 2003;47(1):231–7.
7. WHO Africa. World malaria report 2019 [Internet]. WHO Regional Office for Africa. 2019. Available from: https://www.who.int/news-room/fact-sheets/detail/malaria
8. Gendrot M, Fawaz R, Dormoi J, Madamet M, Pradines B. Genetic diversity and deletion of Plasmodium falciparum histidine-rich protein 2 and 3 : a threat to diagnosis of P . falciparum malaria. Clin Microbiol Infect [Internet]. 2019;25(5):580–5. Available from: https://doi.org/10.1016/j.cmi.2018.09.009
9. Institute EPH. Ethiopia national MIS 2015. 2015;
10. President US, Initiative M. PRESIDENT ' S MALARIA INITIATIVE ETHIOPIA. 2019;
11. Jamil KF, Pratama NR, Marantina SS, Harapan H, Kurniawan MR, Zanaria TM, et al. Allelic diversity of merozoite surface protein genes (msp1 and msp2) and clinical manifestations of Plasmodium falciparum malaria cases in Aceh , Indonesia. Malar J [Internet]. 2021;1–12. Available from: https://doi.org/10.1186/s12936-021-03719-w
12. Soe TN, Wu Y, Tun MW, Xu X, Hu Y, Ruan Y, et al. Genetic diversity of Plasmodium falciparum populations in southeast and western Myanmar. Parasites and Vectors.

2017;10(1):1–6.

13. Bharti PK, Shukla MM, Sharma YD, Singh N. Genetic diversity in the block 2 region of the merozoite surface protein-1 of Plasmodium falciparum in central India. Malar J. 2012;11:1–7.

14. Khaminsou N, Kritpetcharat O, Daduang J, Charerntanyarak L, Kritpetcharat P. Genetic analysis of the merozoite surface protein-1 block 2 allelic types in Plasmodium falciparum clinical isolates from Lao PDR. Malar J. 2011;10:1–14.

15. Razak MRMA, Sastu UR, Norahmad NA, Abdu-Karim A, Muhammad A, Muniandy PK, et al. Genetic diversity of plasmodium falciparum populations in malaria declining areas of Sabah, East Malaysia. PLoS One. 2016;11(3):1–22.

16. Gosi P, Lanteri CA, Tyner SD, Se Y, Lon C, Spring M, et al. Evaluation of parasite subpopulations and genetic diversity of the msp1 , msp2 and glurp genes during and following artesunate monotherapy treatment of Plasmodium falciparum malaria in Western Cambodia. 2013;

17. Oyedeji SI, Bassi PU, Oyedeji SA, Ojurongbe O, Awobode HO. Genetic diversity and complexity of Plasmodium falciparum infections in the microenvironment among siblings of the same household in North-Central Nigeria. Malar J [Internet]. 2020;19(1):1–10. Available from: https://doi.org/10.1186/s12936-020-03415-1

18. Chen JT, Li J, Zha GC, Huang G, Huang ZX, Xie D De, et al. Genetic diversity and allele frequencies of Plasmodium falciparum msp1 and msp2 in parasite isolates from Bioko Island , Equatorial. Malar J [Internet]. 2018;1–9. Available from: https://doi.org/10.1186/s12936-018-2611-z

19. Metoh TN, Chen JH, Fon-Gah P, Zhou X, Moyou-Somo R, Zhou XN. Genetic diversity of Plasmodium falciparum and genetic profile in children affected by uncomplicated malaria in Cameroon. Malar J [Internet]. 2020;19(1):1–15. Available from: https://doi.org/10.1186/s12936-020-03161-4

20. Badoum ES, Bougouma EC, Sombie S, Sermé SS, Yaro JB, Diarra A, et al. Relationship between human genetic factors and Plasmodium falciparum genetic diversity of msp1 , msp2 and glurp in a malaria endemic area of Burkina Faso. 2019;4:1–9.

21. Nabet C, Doumbo S, Jeddi F, Konaté S, Manciulli T, Fofana B, et al. Genetic diversity of

Plasmodium falciparum in human malaria cases in Mali. Malar J. 2016;15(1):1–10.

22. Oyebola K M, Idowu T E. Genetic Diversity and Complexity of Plasmodium Falciparum Infections in Lagos, Nigeria. J Bacteriol Parasitol. 2014;5(4):2–5.
23. Abdel Hamid MM, Mohammed SB, El Hassan IM. Genetic diversity of Plasmodium falciparum field isolates in Central Sudan inferred by PCR genotyping of merozoite surface protein 1 and 2. N Am J Med Sci. 2013;5(2):95–101.
24. Mayengue PI, Ndounga M, Malonga FV, Bitemo M, Ntoumi F. Genetic polymorphism of merozoite surface protein-1 and merozoite surface protein-2 in Plasmodium falciparum isolates from Brazzaville , Republic of Congo. 2011;(February 2006):1–7.
25. Abukari Z, Okonu R, Nyarko SB, Lo AC, Dieng CC, Salifu SP, et al. Population Structure of P . falciparum Parasites and Low Malaria Transmission Settings of Ghana. :1–14.
26. Mahdi Abdel Hamid M, Elamin AF, Albsheer MMA, Abdalla AAA, Mahgoub NS, Mustafa SO, et al. Multiplicity of infection and genetic diversity of Plasmodium falciparum isolates from patients with uncomplicated and severe malaria in Gezira State, Sudan. Parasites and Vectors [Internet]. 2016;9(1):1–8. Available from: http://dx.doi.org/10.1186/s13071-016-1641-z
27. Mustafa SO, Hamid MMA, Aboud MA, Amin M, Muneer MS, Yasin K, et al. Genetic diversity and multiplicity of Plasmodium falciparum merozoite surface protein 2 in field isolates from Sudan [version 1 ; peer review : 1 not approved]. 2020;2–10.
28. Touray AO, Mobegi VA, Wamunyokoli F, Herren JK. Diversity and Multiplicity of P . falciparum infections among asymptomatic school children in Mbita , Western Kenya. Sci Rep [Internet]. 2020;1–8. Available from: http://dx.doi.org/10.1038/s41598-020-62819-w
29. Mohammed H, Hassen K, Assefa A, Mekete K, Tadesse G, Taye G, et al. Genetic diversity of Plasmodium falciparum isolates from patients with uncomplicated and severe malaria based on msp-1 and msp-2 genes in Gublak, North West Ethiopia. Malar J [Internet]. 2019;18(1):1–8. Available from: https://doi.org/10.1186/s12936-019-3039-9
30. Mohammed H, Kassa M, Mekete K, Assefa A, Taye G, Commons RJ. Genetic diversity of the msp-1, msp-2, and glurp genes of Plasmodium falciparum isolates in Northwest Ethiopia 11 Medical and Health Sciences 1108 Medical Microbiology. Malar J [Internet]. 2018;17(1):1–8. Available from: https://doi.org/10.1186/s12936-018-2540-x

31. Mohammed H, Mindaye T, Belayneh M, Kassa M, Assefa A, Tadesse M, et al. Genetic diversity of Plasmodium falciparum isolates based on MSP-1 and MSP-2 genes from Kolla-Shele area, Arbaminch Zuria District, southwest Ethiopia. Malar J. 2015;14(1):1–7.
32. Mohammed H, Kassa M, Assefa A, Tadesse M, Kebede A. Genetic polymorphism of Merozoite Surface Protein-2 (MSP-2) in Plasmodium falciparum isolates from Pawe District , North West Ethiopia. 2017;2:1–11.
33. Status C. Genetic diversity and genotype multiplicity of Plasmodium falciparum infection in patients with uncomplicated malaria in Chewaka district , Ethiopia. :1–21.
34. Talie A. Prevalence of malaria and associated risk factors among asymptomatic migrant laborers in West Armachiho District , Northwest Ethiopia. 2018;95–101.
35. Baidjoe A, Stone W, Ploemen I, Shagari S, Grignard L, Osoti V, et al. Combined DNA extraction and antibody elution from filter papers for the assessment of malaria transmission intensity in epidemiological studies. Malar J. 2013;12(1):1–9.
36. Mwingira F, Nkwengulila G, Schoepflin S, Sumari D, Beck HP, Snounou G, et al. Plasmodium falciparum msp1, msp2 and glurp allele frequency and diversity in sub-Saharan Africa. Malar J. 2011;10:1–10.
37. Snounou G, Zhu X, Siripoon N, Jarra W, Thaithong S, Brown KN, et al. Biased distribution of msp1 and msp2 allelic variants in Plasmodium falciparum populations in Thailand. Trans R Soc Trop Med Hyg. 1999;93(4):369–74.
38. Kiwuwa MS, Ribacke U, Moll K, Byarugaba J, Lundblom K, Färnert A, et al. Genetic diversity of Plasmodium falciparum infections in mild and severe malaria of children from Kampala , Uganda. 2013;1691–700.

ANNEXES

Annex .DNA extraction protocol for Dried Blood Sample (DBS) by Chelex method

Purpose: To obtain malaria parasite DNA from dried blood spot on the standard filter paper (Whatman filter paper) for molecular analysis

Principle: Malaria parasite DNA from the DBS is extracted by heating samples in a suspension of 5%-30% Chelex. Heating in water bath at 96 0C in an alkaline suspension disrupts the cell membrane and breaks down proteins including heat-labile enzymes while Chelex solution, an ion chelator, limits destruction of the DNA by inactivating nucleases and chelating heavy metals that may damage parasite DNA.

Required material

Puncher /scissor

CD marker

Forceps

PBS-container

1.5 ml microcentrifuge tubes

Chelex- container

0.5 ml microcentrifuge tubes

Sealer of DNA extraction plate(optional)

Single channel Micropipette (1-20µl, 100-200 µl, 1000 µl)Shaker (optional)

Micropipette Tip (20 µl, 200 µl, 1000 µl)Bunsen burner & Lighter/Match (optional)

Analytical balanceTissue paper (optional)

Water bathAspirator (Optional)

MicrocentrifugeRefrigerator

Plate centrifuge (Depending on the workload)

Required chemicals and Reagent

- PBS solution (Appendix-I)
- 0.5% Saponin/PBS (Appendix-II)
- 6% Chelex (Appendix-III)

Condition to be considered before starting the procedure

- Clean the work bench by disinfectant(1:10 diluted beach) and 70% alcohol
- Collect the necessary material
- Prepare required working reagent if not available (NB. Chelex reagent should be made fresh each day based on the need)
- Cover the work bench with M-tork(Tissue paper)
- Arrange the DBS/sample, disinfectant and/ or Bunsen burner in the appropriate place

Procedure

Day 1.

1. Enter sample information in excel (Extraction list) or logbook. Specify date of extraction
2. Prepare the work area (Disinfect the area and cover the bench with tissue paper)
3. Label 1.5 ml microcentrifuge tubes or well of a 96-well microtiter plate with the filter paper sample ID (label both the lid and the side of the tube)
4. Disinfect the puncher / scissor and forceps before and after punching by dipping in 70 % Alcohol and passing through Bunsen flame
5. Cut 2-3 pieces of 3 mm x 3 mm or punch a 3 mm-5mm disk (holds approx. 3-5 □l of dried blood) from the filter paper. Cut in small strips before put into a 1.5ml tube.
6. Put immediately the small pieces of filter paper into the corresponding 1.5 microcentrifuge tube or well of a 96-well microtiter plate using forceps
7. Add 1ml of PBS containing 0.5 % Saponin (950ul PBS + 50ul 10% Saponin) into the corresponding 1.5ml microcentrifuge tube or 96-well microtiter plate

NB. Ensure filter papers are soaked in buffer (PBS)

8. Mix well and make sure filter papers are completely immersed inside buffer (PBS)
9. Incubate microcentrifuge tube or 96-well microtiter plate at 4C for 1- 2 nights depending on color change

Day 2:

9. Vortex and then centrifuge the mixture at 13,000 rpm for 10 mins at room temperature

10.Discard the liquid content using P1000 pipettor

11. Vortex and add 1ml of PBS and leave at 4C for 30 mins

12. Centrifuge at 13,000 rpm for 5 mins

13. Discard as much liquid as possible using P1000 pipette tips

14. Add 300ul 6 % Chelex in TE buffer

15. Vortex and incubate at 95C for 12 mins. Open cap to release pressure after 2 min and vortex every 3 mins

16. Centrifuge at 13,000 rpm for 8 mins

17. Transfer supernatant into clean labeled 1.5 ml centrifuge tubes

18. Centrifuge again at 13,000 rpm for 1 min

19. Transfer liquid to clean labeled 0.5 ml centrifuge tubes. Avoid chelex particles reside at the bottom. Volume should be ~250ul for each tube

20. Put finished tubes in a white cardboard box. Good quality DNA should be of clear color, may be light yellowish sometimes

21. Label box with a consistent labeling system and then store box at -20C

Appendix-I: PBS solution

PBS Tablet……….one Tablet

Distilled water…….200ml

Procedure for preparation of PBS solution

1. Measure 200ml distilled water in measuring cylinder
2. Transfer 200ml distilled water in the flask and add one tablet PBS
3. Dissolve the tablet using electronic stirrer
4. Autoclave dissolved PBS solution

Appendix-II: 0.5% saponin /PBS solution

Saponin powder……..0.5gm

PBS solution…………100ml

Appendix-III: 6% Chelex

1. Chelex 100resine…………………6gm
2. DNase/ RNase free water ………. .100ml

Note: Depending on the number of DBS/sample size required amount of PBS solution, 0.5% saponin /PBS solution and 6% Chelexcan be prepared proportionally.

Note: If required 20 % chelex, add 10 ml distilled water to 2 g Chelex

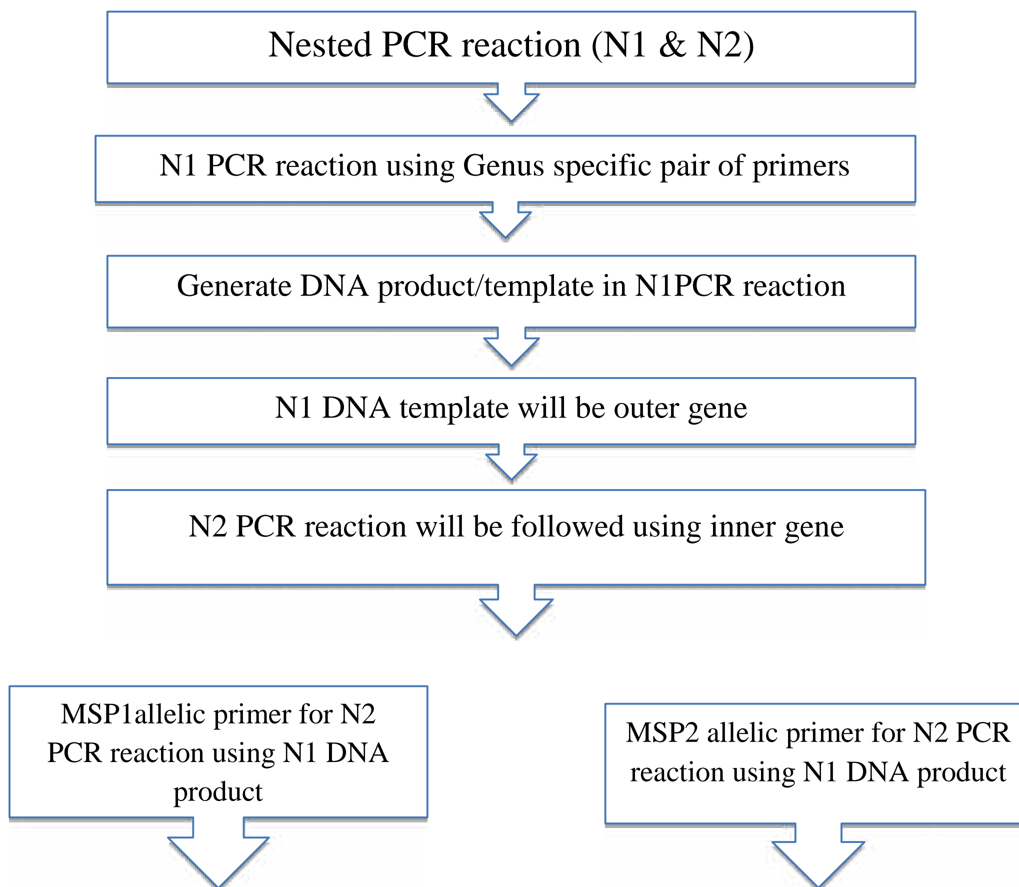
Annex . Nested PCR (N1 and N2) amplification for *MSP1 AND MSP2* DNA template

1. Purpose: Detection and identification of*MSP1 and MSP2* from DBS DNA template that occur very few amount

i.e. The efficiency of the assay is markedly improved when nested PCR strategy employed.

i.e. Used to increase the specificity of DNA amplification

2. Principle of Nested PCR: Two round of amplification are carried out. In the first round of PCR (Nest1), one pair of primers is used to generate DNA products, which will be the target/ serving as a template for the second reaction. Using one ('hemi-nesting') or two different primers whose binding sites are located (nested) within the first set, thus increasing specificity.



Identification and detection of *MAD20, K, and RO33*

Identification and detection of *FC27, 3D7*

Required dedicated area for Molecular laboratories

Laboratories should have at least three dedicated areas for each stage of the procedure to avoid false positive result:

1st .**PCR Master mix set-up area:** no nucleic acids in this area (PCR reagents only)

2nd **DNA template area:** area to handle extracted DNA (Processing of sample, Nucleic acid isolation and addition of DNA template to master mix aliquant)

3rd Amplification and product visualization area: Thermo cycle, Gel electrophoresis and Amplified DNA template handling area

3. **Specimen/sample:-**

➢ DNA template extracted from DBS using chelex method

- 4µl Extracted DNA for N1 PCR (DNA template must be at 4^0C to run PCR)
- 2µl of N1 DNA product for N2 PCR
- Negative control
- Positive control

Required PCR equipment

- Thermocycle
- Micropipette (single and Multichannel, with different size)
- Micropipette tip (with different size)
- PCR tube / plate
- Ependrof tube (1-2ml for master mix preparation)
- Mini-centrifuge
- Plastic sealer for PCR plate
- Permanent marker

Required PCR Reagent

1. PCR grade water
2. Master Mix

3. Nested PCR primers

Procedure to run Nested PCR

A.First Round PCR amplification (Nest 1) (Snounou et al. 1993)

1. Go to PCR master mix set-up area, add all PCR reagents except the template DNA into one tube; this is the "Master Mix". See Table 2 for formulation.

2. Aliquot 16µL of Master Mix into PCR plate/ PCR tube.

3. Add 4µL DNA template product into the PCR Plate / PCR tube

Plate. / PCR tube (Note: DNA template should be at 4 ^{0}C)

4.Load samples into thermal cycler. See Table 4 for N1 PCR program thermal cycler

5.N1 amplicon product should be put at 4 ^{0}C until N2 proceed

B. Second (Nest 2) round amplification (Snounou et al. 1993)

1. Go to **PCR master mix set-up area**, add all PCR reagents except the template DNA(N1 product) intoone tube; this is the "Master Mix".

2. Aliquot 18µL of Master Mix into PCR plate/ PCR tube.

3. Move to the DNA template area and add 2µL N1 DNA product into the PCR Plate / PCR tube

4.Move to amplification and product visualization area, load samples into thermal cycler.

Table 1: PCR program (N1) Snounou et al. 1993

	PCR program (N1)			
Step/cycle	**Time**	**Temperature(^{o}C)**	**Purpose**	
1st step	10min	95	Initial denaturing	
2nd step	60sec	95	Denaturing	
3rd step	60sec	58	Annealing	**x35**
4th step	90sec	72	Extending	
5th step	10min	72	Extending	
		10	To hold N2 product	

Table 2: PCR program (N2) Snounou et al. 1993

	PCR program (N2)			
Step/cycle	**Time**	**Temperature(^{o}C)**	**Purpose**	
1st step	10min	95	Initial denaturing	
2nd step	60sec	95	Denaturing	
3rd step	60sec	58	Annealing	

4th step	90sec	72	Extending	**x30**
5th step	10min	72	Extending	
		10	To hold N2 product	

C. Visualization of Amplified DNA on a 2% Agarose Gel

1. Move to gel electrophoresis area.

2. Make a 2% (w/v) agarose gel (2g per 100 mL of 0.5X TAE)(Appendix III)

A. Total volume will be dependent on electrophoresis apparatus size (Example: 6g per 300 mL of 0.5X TAE used to perform four gel in one electrophoresis tray)(Appendix III)

B. Put in microwave for 3minute to dissolve agarose, cool agarose to ~50°C (tape water to cool)

C. Add 6µl of ethidium bromide into 300ml of agarose gel solution (i.e. 2µl ET Br for 100ml)

D. Arrange /adjust gel casting tree to load 300m of 2% agarose gel

- Balance gel casting tree using blue eyes
- Put comb having different size/Number lane by jumping two raw /space (no of the gel is depending on the sample size)

E. Pour 2% agarose gel ethidium bromide into gel casting tree

F Allow gel to cool (40 0C) and form mold (solidify) for 20minute completely before removing comb (Remove the comb carefully)

G. After removing the comb, solidified agarose gel with casting tree immersed into electrophoresis chamber/tanker filled with 1X TBE buffer in a manner that the surface of gel is fully covered by the TBE buffer

3. Load Amplicon product and molecular ladder into the lane

1st: 5µl load molecular ladder on 1st lane

2nd: Load 10µl of amplicon product (sample) starting from 1st lane (volume of Amplicon product dependent on well size)

3rd: load at least one positive control, one negative control and blank in the last lane respectively

4. Subject agarose gel to electrophoresis

1st: Close the loaded casting tree with negative and positive electrode properly

2nd: Run electrophoresis by adjusting at 120 voltage and 400 ampere for 60minute (Voltage and time is dependent on gel size)

Note: Run samples until the pink dye (~25 bp) has migrated at least 2/3 of the way through the gel.

5. Visualize amplicon product after electrophoresis by gel doc.system

1st : After 60 minute of running electrophosis, switch off the elelctrophosis chamber and take out the gel and cut the gel step by step(1st to 2nd gel)

2nd. Switch on the reader and also computer

Note: Sample will be visualized under UV-light and image will be taken using computer installed software connected to the Bio-RAD.

3rd: Take 1st gel (and 2nd gel up to 4th gel step by step) and put it at the center of gel doc system 4th: close gel doc system after avoiding bubble

5th: Go to computer connected to this gel doc system and click the installed software as follow:-

A. First click Quantity one (file name of software) from desk top,

B. Second Click Trans UV,

C. Third click Auto

D. Fourth click freeze just after 1.5-2 second of running.

E. Save the file of each gel in the computer by code no of the sample

Interpretation of gel lane and editing the lane

1st: First look for control lane

- Band should be observed in the three positive control lane
- Band should not be appeared in the three negative control lane

2nd: If the control lane is valid, interpret the sample using molecular ladder.

- The expected amplicon product of Genus Plasmodium is 1200bp
- The expected amplicon product of *P.falciparum* is 205bp (snounoun et al 1993)
- The expected amplicon product of *P.vivax* is 120bp (snounoun et al 1993)

3rd: After interpretation, it is possible to edit the gel lane (color& contrast) and also label the sample, control and Molecular ladder of the lane by taking in the excel.

Appendix-I: Preparation of 10x Stock TBE solution

To prepare 1000ml TBE stock (10X) solution

- Borric acid……….55g
- Tris (triose base)….108g
- 0.5HNa2EDTA……40ml
- Distilled water……..960ml

Procedure for preparation of 10x Stock TBE solution

1st step: Add 800ml distilled water in the bottle

2nd step: Add 108g Tris and 55g Borric acid in bottle which contain distilled water and stir it by magnetic stirrer to dissolve the chemical

3rd: Add 40ml 0.5 HNa2EDTA(pH;8.0) or 7.5g of Na2Co3

4th: Adjust the total volume to 1000ml (just by adding 160ml of distilled water)

5th: Filter by 0.2µm filter

6th: Store at room temperature

Appendix II: Preparation of 0.5% TBE buffer solution

-0.5 % TBE Buffer will be prepared from 10% TBE buffer stock solution after stirring and filtering using 0.2µm filter

To prepare 1000ml of 0.5% TBE buffer solution

10% TBE Buffer………..50ml

Distilled water…………..950ml

- We have used the following formula to prepare 0.5 % TBE Buffer : C1V1=C2V2,

C1=concentration of the stock=10%, V1=?

C2=concentration of the required TBE buffer =0.5 %,

V2= the required volume to be prepared=1000ml

Appendix III: Preparation of working 0.5% TBE buffer solution directly from the chemical (Note Alternative Procedure for Appendix II)

To prepare 1000ml of 0.5% TBE buffer solution

Follow the following Procedure:

1st step: Add 800ml distilled water in the bottle

2nd step: Add 10.8g Tris and 5.5g Borric acid in bottle which contain distilled water and stir it by magnetic stirrer to dissolve the chemical

3rd: Add 4ml 0.5 HNa2EDTA(pH;8.0) or 0.75g of Na2Co3

4th: Adjust the total volume to 1000ml (just by adding 160ml of distilled water)

5th: Filter by 0.2µm filter

6th: Store at room temperature

Appendix IV: preparation of 2% agarose

- 2% (w/v) agarose gel means 2g per 100 mL of 0.5% TBE

To make 300ml of 2% agarose gel

2g 100ml = 6g

? 300ml

Agarose powder……..6g

0.5% TBE……………300ml

1. Measure 6g of agarose powder and add into 300ml into 0.5% TBE buffer in the flask

2. Mix agarose –TBE solution and boiled for 3minute in microwave oven and cool it with tape water

ANNEX : Declaration

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

M.Sc. candidate: TsegayeChekolAnagaw (B.Sc., MSc candidate)

Signature: ___________________

Date of submission: ___________________

This proposal has been submitted with our approval as advisors.

Advisor: Dr. MistireWolde (MSc, PhD)

Signature: _____________________

Date: _____________________

Place: Addis Ababa, Ethiopia.

Advisor: BirukZerfu (MSc, PhD candidate)

Signature: ____________________

Date: ____________________

Place: Addis Ababa, Ethiopia.