Genetic diversity and multiplicity of infections of *Plasmodium falciparum* clinical isolates from Adama and its surroundings, Oromia, Ethiopia

Temesgen File Huluka

A Dissertation Submitted to the Department of Applied Biology, School of Applied Natural Sciences

Presented in Fulfilment of the Requirements for the Degree of Doctor of Philosophy in Biotechnology (Specialization in Health Biotechnology)

Office of Graduate Studies
Adama Science and Technology University

December, 2021
Adama, Ethiopia

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Declarations and Recommendations

Declaration

I hereby declare that this Dissertation entitled "Genetic diversity and multiplicity of infection of *Plasmodium falciparum* clinical isolates from Adama and its surroundings, Oromia, Ethiopia" is my original work. That is, it has not been submitted for the award of any academic degree, diploma or certificate in any other university. All sources of material used in this thesis have been duly acknowledged through appropriate citations.

_____	_____	_____
Name of student	Signature	Date

Recommendation

I/we, the supervisor (s) of this dissertation, hereby certify that I/we have read and revised the dissertation entitled "Genetic diversity and multiplicity of infections of *Plasmodium falciparum* clinical isolates from Adama and its surroundings, Oromia, Ethiopia". This PhD Dissertation is prepared by **Temesgen File Huluka**; under my/our guidance and submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biotechnology (Specialization in Health Biotechnology). The student has successfully defended his PhD Dissertation and incorporated the comments given by the examining board. Therefore, I/we recommend the submission of the final version of this PhD Dissertation to the department for approval.

Major supervisor	Signature	Date
Co-supervisor	Signature	Date

Approval Page of Ph.D. Dissertation

Approval Page of Ph.D. Dissertation I/we hereby certify that the recommendations and suggestions made by the board of examiners are appropriately incorporated into the final version of the dissertation entitled "**Genetic diversity and multiplicity of infection of *Plasmodium falciparum* clinical isolates from Adama and its surroundings, Oromia, Ethiopia"** by **Temesgen File Huluka**.

Major supervisor	Signature	Date
Co- supervisor	Signature	Date

We, the undersigned, members of the Board of Examiners of the dissertation open defense by **Temesgen File Huluka** have read and evaluated the dissertation entitled "**Genetic diversity and multiplicity of infection of *Plasmodium falciparum* clinical isolates from Adama and its surroundings, Oromia, Ethiopia"** and examined the candidate during open defense. This is, therefore, to certify that the dissertation is accepted for partial fulfillment of the requirement of the degree of Doctor of Philosophy in **Biotechnology (Specialization in Health Biotechnology**)

Chairperson	Signature	Date
Internal Examiner 1	Signature	Date
External Examiner 1	Signature	Date
External Examiner 2	Signature	Date

Finally, approval and acceptance of the dissertation is contingent upon submission of its final copy to the Office of Postgraduate Studies (OPGS) through the candidate's Department Graduate Council (DGC) and School Graduate Committee (SGC

Department Chair	Signature	Date
School Dean	Signature	Date
Postgraduate Dean	Signature	Date

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Abbreviations and Acronyms

AT	Adenine, Thiamine
Bp	Base pair
CNV	Copy number variation
CSP	Circum-sporozoite protein
DBS	Dry Blood Spot
DNA	Deoxyribonucleic acid
FMOH	Federal Ministry of Health
GLURP	Glutamate Rich Protein
GPI	Glycosylphosphatidyl inositol
GWA	Genome wide association
He	Expected heterozygosity
HBsAg	Hepatitis B surface (S) antigen
IE	Infected Erythrocytes
IERB	Institutional Ethical Review Board
IRS	Indoor Residual Spray
iRBCs	infected red blood cells
ITN	Insecticide Treated Net
Indel	Insertion-deletion
kDa	kilo- Dalton
LAMP	Loop mediated isothermal amplification
Mb	Mega base
MM	Master mix
MOI	Multiplicity of Infection
MSP	Merozoite Surface Protein
msp-1 gene	Merozoite surface protein 1 gene
msp-2 gene	Merozoite surface protein 2 gene
MSPDBL1/2	Merozoite surface protein Duffy binding ligand 1/2
N1	Nested 1
N2	Nested 2

NASBA	Nucleic acid sequence-based amplification
NGO	Non-Governmental Organization
NGS	Next generation sequencing
PCR	Polymerase Chain Reaction
Pfmp1	*Plasmodium falciparum* membrane protein 1
PV	Parasitophorous Vacuole
RBC	Red Blood Cells
RNA	Ribonucleic acid
SAC	School age children
SD	Standard Deviation
SNP	Single nucleotide polymorphism
SOP	Standard Operating Procedure
SSA	Sub Saharan Africa
UN	United Nation.
WBC	White Blood Cells
WHO	World Health Organization

Abstract

Genetic diversity of P. falciparum can be determined by genotyping of the polymorphic regions like block 2 region of merozoite surface protein (msp-1) and block-3 region of merozoite surface protein (msp-2). Understanding of these polymorphic genes of P. falciparum can inform mechanisms underlining disease pathology, identification of the circulating parasite clones, transmission intensity and potential deficiencies and/or strengths of the ongoing malaria control and elimination efforts in the locality. Detailed understanding of local genetic polymorphism is an input to pave the way for better management, control and elimination of malaria. The aim of this study was therefore to explore the status of these polymorphic genes and their implications in Adama and its surrounding. For this purpose, a total of 171 DBS sample from uncomplicated P. falciparum patients was collected from Adama, Modjo, Wonji, Awash Malkasa, and Olanciti, through September 2019 to August 2020. From these, 139 and 148 clinical isolates were successfully amplified for msp-1 and msp-2 genes, respectively, using specific primers. Nested PCR amplification was conducted targeting K1, MAD20, and RO33 alleles for msp-1 and 3D and FC27 alleles for msp-2 gene followed by gel electrophoresis for fragment analysis. Based on the detection of a PCR fragment, infections were classified as monoclonal or multiple infections. Accordingly, 19 different size polymorphism of msp-1 gene were identified in this study, with 67(48.2 %) MAD20, 18 (13 %) K-1 and 18 (13 %) RO33 allelic family. Whereas, the multiple infections were 21(15 %), 8 (5.8 %), 4(2.9 %), 3(2.2 %) for MAD20+K-1, MAD20+RO33, K-1+ RO33, and MAD20+K-1+ RO33, respectively. The overall Multiplicity of infection (MOI) was 1.42 and the expected heterozygosity (He) was 0.65. Moreover, 17 different polymorphic forms of msp-2 allelic fragments were detected in the study area. A total of 47 (31.8%) and 41(27.7%) were detected for 3D7 and FC27 allelic family, respectively. Furthermore, for msp-2 gene: the multi-clonal allele type accounted for 60(40.5%). The overall MOI and expected heterozygosity were 1.46 and 0.68, respectively. The study also revealed presence of ≥ 2 clones of infection in patient isolates with the same allele in both markers (msp-1 and msp-2) genes. Therefore, the study sheds light on the presence of moderate level of genetic diversity of P. falciparum in the study area, compared with similar study in different parts of Ethiopia, demanding further scale up of the ongoing control and elimination efforts.

***Keywords**: Expected heterozygosity, genetic diversity, msp-1, msp-2, Multiplicity of infection, P. falciparum.*

1. INTRODUCTION

1.1 Background

Malaria is a parasitic infectious disease that show real spatial heterogeneity globally (Snow *et al*., 2005), regionally (Gemperli *et al*., 2006), and at local scales (WHO, 2017). A number of suitable environmental predictors and climatic factors for its transmission attributes to the malaria burden. Malaria transmission from human to human takes place by the bite of infected female Anopheles mosquito "malaria vectors", which bites mainly between dusk and dawn. Its transmission intensity depends on factors related to the vector, the parasite, the host and environment (WHO, 2017). World Health Organization (WHO) reported that; in 2019, the number of malaria cases were 229 million, out of which 409,000 deaths recorded. More than 90% of all malaria cases and death were only from sub-Saharan Africa (SSA) (WHO, 2020). Only 15 countries, all from sub-Saharan Africa except India accounts for 80% of the global malaria burden (Ariey and Gay, 2019). Factors that contributed to such highest malaria episode in SSA are: poverty (Sonko *et al.*, 2014) and the highest burden is due to *P. falciparum,* the deadliest of all human malaria parasite (WHO, 2014; WHO, 2017). Moreover, suitable-micro ecological factors exist in SSA, that favor mosquito to live longer and allows the parasite to complete its development (Ariey and Gay, 2019). Besides, the most effective malaria vector, *Anopheles gambiae* is widespread in Africa and most difficult to control (Lo *et al.*, 2017). The local weather conditions favor malaria transmission to occur year round in some areas or seasonal in others.

About 68% of Ethiopian population inhabits in 75% of the countries land mass that is malarious (FMOH, 2010, FMOH, 2020; Solomon *et al*., 2020) . In 2018 alone, the number of confirmed malaria cases reported was 1,206,892. Of this 883,886 (69.2%), and 181,964 (30.8%), were *P. falciparum* and *P. vivax,* respectively (FMOH, 2020; Solomon *et al*., 2020). In Ethiopia, there are two seasons of malaria transmission: the major and minor seasons. The major season of transmission occurs between September to December in most areas following the main rainy season from June to August. The minor transmission season occurs from April to May, after a short rainy season from February to March (FMOH, 2010). These bi-annual transmission pattern overlap with the major agricultural seasons and incapacitate productivity. Consequently, malaria is not only, a cause for socio-economic burden, but also the cause and outcome of the vicious circle of poverty fueling each other. Consequently, malaria transmission occurs indoors, during the

evening hours, but its transmission may also occur in outdoors during the evening time work or other social (Mendis *et al*., 2001), and economic activities in both rural and urban areas.

The proportion of Africans living in cities is rapidly rising and projected to reach 50% by 2030, profoundly altering the epidemiology of malaria (Siri *et al*., 2008). As the world is becoming urban, malaria is no longer considered just a rural issue alone. Due to fast rate of urbanization in Ethiopia, there is frequent emergence of small and medium- sized cities with a great mismatch between urbanization rate and infrastructure development. This evolving conditions not only favors mosquito breeding but also, adaptation of malaria vector in the changing urban ecosystem. In addition, the socio-economic disparity of the urban community to afford protection, and sometimes misdiagnosis and treatment due to the difficulty in distinguishing clinical malaria from other febrile illness with the presumption that urban development reduces vector breeding and malaria transmission. As reported in some African cities' malaria transmission persists in urban areas and in some cases at even higher levels than its surrounding rural counterpart (De Silva and Marshall, 2012). Due to the potential communication nodes of *P. falciparum* population between rural and urban localities, genetic diversity and multiplicity of infection (MOI) observed could reflect the dynamics of the parasite clones circulating in the area. Similarly, the flow of parasite populations between urban and rural settings might be important to explain the differences in genetic diversity and eventually transmission status the parasite population. Therefore, for effective implementation of malaria control and elimination in rural and urban setting, comprehensive molecular epidemiological study of malaria demands due attention in Ethiopia. In this regard, very limited molecular data were available in the country. No data is yet available in malaria endemic region of central Ethiopia like Adama and its surroundings that could explain genetic diversity of *P. falciparum* and its temporal and spatial dynamics of the parasite clones in the region.

The aim of the present study was, therefore, to describe genetic diversity and MOI of *P. falciparum:* the most virulent, highly polymorphic and predominant malaria parasite primarily targeted in malaria control and eventual elimination. The study focuses on polymorphic markers of merozoite surface protein (*msp-1 and msp-2*) genes of *P. falciparum* and MOI from sample collected from three districts around Adama, including Adama town administration. The study generated comprehensive data on the status of genetic diversity, MOI, the spatial and temporal

features of its transmission dynamics including urban rural settings. The findings of the study would enable us to assess effectiveness of the prevailing malaria control and elimination undertakings in the study area. From the present study, valuable recommendations were generated that could supplement the ongoing malaria control and elimination efforts at local, regional and national levels.

1.2 Statement of the problem

In Ethiopia, areas less than 2000 meters above sea level are considered malarious (FMOH, 2020; FMOH, 2010). Due to rigorous efforts to control malaria, the disease has been pushed back in both rural and urban dwellings. Areas like Adama, Modjo, Wonji, Awash Malkasa, and Olanciti, are located within the escarpment of the Great Rift Valley areas of central Ethiopia. In addition to their physical location, different micro-ecological factors favored mosquito breeding and malaria transmission. It was reported that, frequent stagnation of water after rainy seasons, characteristic features like bushy gorges, ditches, shanty and slum dwelling, poor tidiness were very common in Adama town (File and Dinka, 2020). Moreover, vegetation covered residential areas, thrown away broken equipment, possibly accompanied by in appropriate or no use of insecticide treated net (ITN), and indoor residual spray (IRS), are some of the factors that favor mosquito breeding and malaria transmission in Adama town (File and Dinka, 2020; File *et al.,* 2019). Similar scenario and micro ecological factors also exists in the surrounding districts. Despite rigorous effort at national and local levels to control and eliminate malaria burden, the five year trend analysis of malaria cases in the study area has shown only slight reduction of slide positivity rate from 16.3% in 2016 to 10.1% in 2020 (File and Chala, 2021). Besides rapid and unprecedented urbanization rate, going hand in hand with mismatch with infrastructure development in the region might have profound implications on the epidemiology and control of malaria, in contributing the relative disease burden among urban areas and the surrounding rural counterparts.

Due to the existence of micro-ecological factors that favor malaria, the present low transmission settings characterized by high level of spatial heterogeneity of the disease occurrence with a high potential of its resurgence. Evidence on genetic diversity and the status of MOI of *P. falciparum* is informative on the potential emergence and spread of drug resistance strains. Moreover, the status of genetic diversity of *P. falciparum* indicates the level of effectiveness of drugs presently in use for the treatment, and the existence of potential transmission hot spots in the study areas.

Therefore, there is a need for systematic molecular epidemiological approach to study and characterize the genetic diversity of *P. falciparum* in urban and the surrounding rural areas. Indeed, the findings of the present study would provide vital molecular evidence on the progress towards malaria control and eventual elimination in the country. In Ethiopia, there is paucity of evidence on the situation of urban and rural transmission dynamics of malaria as to whether the same parasite clones are circulating both in urban and rural areas or whether distinct parasite clones are circulating in urban and rural areas. In this regard, although few studies are available, they were mainly based the traditional epidemiological study approach. However, in the present study, major emphasis was made to explore genetic diversity and MOI in urban and its surrounding rural areas by genotyping of the most polymorphic markers of *P. falciparum (msp*-1 and *msp*-2 genes). This could enable us to generate comprehensive data, which will be an input in planning for malaria control and elimination strategy.

1.3 Objectives

1.3.1 General Objective

The general objective of the study was to explore polymorphisms of *Plasmodium falciparum* merozoite surface protein 1 and 2 genes and multiplicity of infections in clinical isolates from Adama and its surroundings.

1.3.2 Specific Objectives

- To examine parasitaemia level with age and *msp*-1 and *msp*-2 allelic variants of *falciparum* clinical isolates.
- To determine genetic diversity of block 2 region of *msp*-1 allelic families of *falciparum* clinical isolates.
- To determine the genetic diversity of block 3 region of *msp*-2 allelic families of *falciparum* clinical isolates, and
- To examine the status of multiplicity of *falciparum* infections isolated from symptomatic patients in the study area.

1.4 Significance of the study

Pillar one of the global technical strategy for malaria (2016-2030), supporting element 1, stated "harnessing innovation and expanding research" (Ariey and Gay, 2019). Therefore, to accelerate efforts for malaria elimination, examining and sharing local scenario of molecular epidemiology

of malaria parasite with major emphasis on genetic diversity and MOI is considerably important. The status of MOI enables us to evaluate the spatial heterogeneity in malaria transmission and the potential transmission "hotspots" for malaria resurgence. By evaluating the situation of genetic diversity related to *msp*-1, and *msp*-2 *genes* and MOI of *P. falciparum*, identification of clones circulating in both urban and rural areas, and whether certain parasite strains are common in urban setting or vice versa, the study under report would generate specific recommendations on the management of malaria in the study areas. The knowledge gained on such local molecular evidence is informative for targeted malaria intervention. The community, healthcare providers, and policy makers could be benefited from the findings of the study to design tailored intervention strategies to enhance malaria prevention, control, and elimination efforts. The study would also provide comprehensive molecular data on urban malaria and its surrounding counterpart, which could serve as a base line evidence for related study. Together with similar research works, the finding augments the Ethiopian national malaria program monitoring and evaluation plan for its control and eventual elimination strategy.

1.5 Ethical Consideration

This research work conducted after securing permission and approval of Adama Science and Technology University, Institutional Ethical Review Board (IERB), through certificate reference number RECSoANS/BIO/01/2019 and consent and permission of Oromia Regional State Health Bureau. In addition, written informed consent obtained from parents or guardian prior to recruitment. The objectives of the study and its benefit were communicated to the relevant health office and the health facilities to be included in the study and patients and guardians. Study participant were well informed to either participate or reject the study at any time. For children less than 18 years of age consent of their parents or legal guardian was requested to include them in the study. After ensuring the consent of the study participant or their parent or guardian; blood sample was collected by finger prick (appendices 8.6, 8.7 and 8.8). After laboratory test result, healthcare workers treated malaria symptomatic patients based on the national malaria treatment guideline.

1.6 Dissemination of the results

The findings of the study were presented to the scientific community through presentations and research conferences. Publications provided to the academic community through peer reviewed

and reputable journals. Hard copies' of the publications will be provided to Oromia Regional State Health Bureau, Adama town administration, and District health offices.

2. LITERATURE REVIEW

2.1 Malaria epidemiology in the context of urbanization

Non-falciparum human malaria parasites include *P. vivax* showing mild infection with broader global distribution (Battle *et al*., 2014). *P. ovale* is endemic to tropical West Africa and rarely causes severe illness or death (Okafor et al., 2022). The Biological property of *P. vivax and P. ovales*'s relapse from their inactive hyponozoite in liver challenges malaria elimination program (Battle, *et al*., 2014). *P. malariae* is widespread in sub-Saharan Africa and the southeast of the Pacific region, its clinical feature is poorly understood (Hawadak *et al*., 2021). *Plasmodium knowlesi* is a simian malaria parasite, which can infect humans via mosquito bites reported from southeast Asia (Amir *et al*., 2018).

The intensity of transmission of malaria depends on factors related to the parasite, the vector, the human host, and the environment. Malaria transmission is stronger in areas where the mosquito life span is longer, allowing the parasite to complete its development in the mosquito, and especially where human density is high, giving more probability to bite humans rather than animals (Ariey and Gay, 2019). According to UN report, the world population is 7.3 billion in 2015, and projected to reach 8.6 billion in 2030. About 90% of this projection will occur in Asia and Africa (UN, 2015). This results not only the growing number of small and medium sized towns, but also the influx of the rural people to the urban and peri-urban areas. Evidences suggests that economic development, improved housing, drainage system of Anopheles breeding sites, household mosquito proofing, expanded personal protection, effective diagnosis and treatment and other factors that exists in urban area have contributed to the recent global decline in malaria incidence (Wilson *et al*., 2015). However, the other side of this reality is that, unplanned urbanization with poor drainage system, presence of standing water collection everywhere due to bad state of roads, poor housing and migration leading to concentrations of the poorest and most vulnerable, making the urban environment itself a social determinant of health (Doumbe-Belisse, *et al*., 2021, Bermudez-Tamayo *et al*., 2016). The greater adaptability of the predominant malaria vector like *Annopheles gambiae*, *Annopheles coluzzii*, and *Anopheles arabiensis* previously shown strong preference to unpolluted water now displays a great adaptation pattern to polluted waters in urban cities and breed in different human-made habitats including containers filled with water, swimming pools, tyre tracks, water tanks and the like (Doumbe-Belisse, *et al*., 2021). Thus,

although malaria is typically considered mainly a problem of the rural poor, this disease has been a concern in urban settings for centuries (Wilson *et al.*, 2015), the adaptation of malaria vector to urban ecology also demands a due attention. Such urban area is therefore, a potential ground for the emerging and re-emerging communicable diseases in general and malaria in particular. Africa's demography is rapidly changing, with an increasing number of people moving to urban areas (De Silva and Marshall, 2012), with poor infrastructure and economic developments. Ethiopia carries a substantial malaria burden, where information on transmission dynamics and spread of drug resistance across the country is scarce (Lo *et al*., 2017). Like other developing countries, urban centers in Ethiopian are characterized by a poorly developed economic base, high level of unemployment and a worrying incidence of poverty and slum habitation (Kassahun and Tiwari, 2012). Consequently, as revealed in some African cities malaria transmission persists and in some cases at even higher levels than in surrounding rural areas (De Silva and Marshall, 2012).

Studies estimated that urban SSA may account for 6–28% of the global malaria burden (Keiser *et al.*, 2004). Thus, as urbanization is increasing, factors that contribute to the urban malaria become more important. Lack of early diagnosis and treatment, together with the emerging drug resistant strains from time to time greatly contributed to the malaria burden. Report shows that in Africa alone; the costs of illness, treatment and premature death from malaria are at least US $12 billion per year that contributes a significant role for poverty (WHO, 2017). *P. vivax* is responsible for most malaria cases in Asia and Latin America, but it is almost absent from most of central Africa, due to absence of Duffy antigen receptor which *P. vivax* uses to invade human erythrocyte. However, what challenged this interpretation is the fact that *P. vivax* infection in Duffy negative people in different part of Africa (Mendis *et al*., 2001). This scenario demands further investigation.

However, in Ethiopia, the two species are (*P. falciparum* and *P. vivax*) are co-endemic accounting for 70 and 30 % malaria cases respectively (FMOH, 2020). Moreover, due to the characteristic feature of malaria endemic settings the parasite could be frequently present in human circulation without causing malaria symptoms. During such scenario, individuals could carry microscopically detectable levels of parasitaemia asymptomatically, providing reservoir of parasite contributing to its persistent transmission. This could be due to the gradual elicitation of humoral immunity against minimal dose of subsequent attacks. The parasite density may fluctuates overtime in chronic

infection. Sub-microscopic carriers might have had higher density infections prior to blood sampling (Shekalaghe *et al.*, 2005). Hyperparasitaemia is relatively more frequent in the relatively non-immune and sometimes in semi-immune individuals with much variability in clinical features.

Delay in parasite clearance by all antimalarial drugs is associated with increasing parasitaemia and hyperparasitaemia (Sowunmi *et al*., 2011). Due to its rapid multiplication, *P. falciparum* infects erythrocytes of any age there is a potential to develop high-grade parasitaemia. Correlation also exists between an individual's asexual erythrocytic-stage parasite density, and clinical severity. Patients with high parasitaemia more likely develop severe and complicated malaria, that could lead to higher case fatality rate (Ali *et al*., 2008). The clinical course of *P. falciparum* malaria varies according to the level of its endemicity. In highly malaria endemic areas of Africa, where transmission of malaria is high, clinical illness normally reaches a peak in children below five years and declines in later ages. In such cases, children are reported to have considerable parasitaemia without febrile illness. On the other hand, in areas where malaria is less endemic cases of severe *P. falciparum* malaria occur in adults, adolescent as well as in older children having symptomatic illness even at lower levels of parasitaemia (Ali *et al.*, 2008). Thus, the relationship between the parasite density and severity of illness due to *P. falciparum* is not straightforward.

Nowadays, instead of the classical epidemiological and parasitological study, molecular approach with high throughput technologies provides a comprehensive data for the correct identification of species, parasite quantification, sensitive detection of the parasite gene, identification of multiplicity of infection/multiple genotype infection, and characterization of the genetic structure of the target gene in the parasite population. The outcome of which, could provide molecular evidence on the spatial and temporal features of the genetic profile of malaria parasite in a given area.

2.2 Life cycle of malaria parasite

The life cycle of malaria parasite (Figure.1), is complex involving cold-blooded insect vector (female anopheles mosquito), and warm-blooded human host. Human infection commonly occurs after the injection of motile sporozoites by female anopheles mosquito. Then, the parasite first enters liver (hepatocytes), where it multiplies and then re-differentiates to generate thousands of exoerythrocytic merozoite forms. That are released into the blood stream after undergoing exoerythrocytic schizogony (Maier *et al.*, 2009), where they invade erythrocytes.

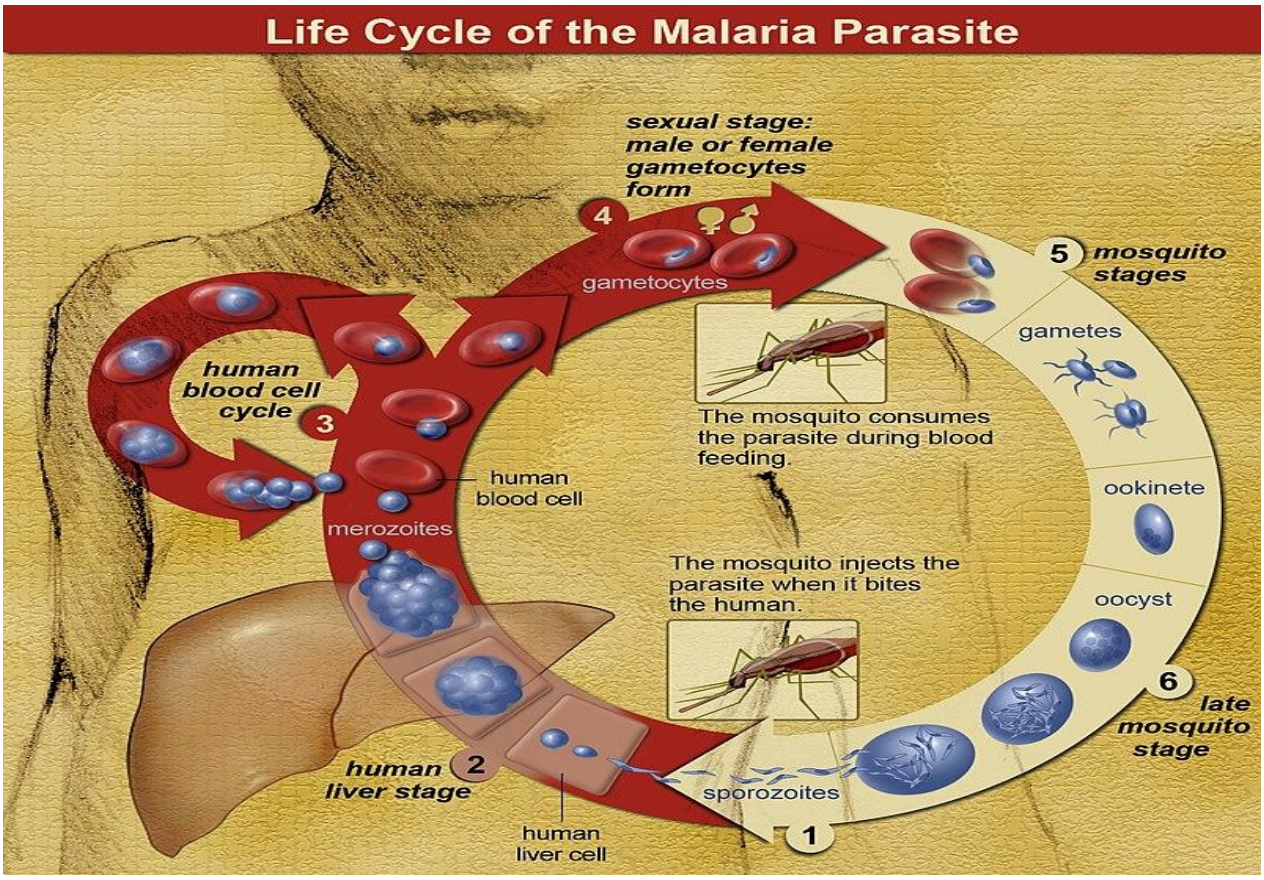


FIGURE 1: **General life cycle of malaria parasite.**

(NIH, 2009)

Then, the parasite grow into trophozoites and mature schizonts, proceeded by erythrocytic schizogony, releasing merozoites, which again re-invade new erythrocytes. Each asexual cycle takes about 48 hours and cell rupture induces periodic waves of fever in patients as the disease progresses (Maier *et al*., 2009). The intra-erythrocytic malaria parasite induces substantial changes in the morphology, physiology and function of the host cell in ways that are designed to promote parasite survival. The changes are initiated by the export of up to 8% of the parasite's gene products to sites in the RBC cytoplasm and at the RBC membrane. These exported proteins interact with the proteins of the RBC membrane and subvert their normal functions (Nilsson *et al.*, 2018; Maier *et al*., 2009). In the process, differentiation of gametocyte from the asexual schizonts of the erythrocytic stage of the parasite takes place due to the activation and expression of the Apatella2-g gene (AP2-G), through epigenetic control by *P. falciparum* heterochromatin protein 1

(PfHP1)(de Jong *et al.*, 2020). A feeding mosquito takes up gametocytes, formed from the asexual blood stage, to the gut where it mature to form male and female gametes.

In mosquito vector, gametogenesis is induced due to the reduction in temperature, increase in pH and exposure to xanthurenic acid (de Jong *et al.*, 2020). In mosquito's mid-gut, plasmodium gametocytes rapidly egress from the host erythrocyte and develop into gametes, followed by fertilization of the gametes forming zygote. Then after, zygote develops to an ookinete and an oocyst and finally sporozoites (Figure.1), that migrate to the salivary glands (Cowman *et al.,* 2012). Sexual reproduction involving malaria parasites of different genotype could also occur when a mosquito feeds on an individual infected with multiple parasite strains or when the same mosquito feeds on more than one human bearing distinct parasite genotypes. This favors genetic recombination and generation of higher diversity that tends to correlate with the transmission intensity (Soe *et al.*, 2017).

2.3 Malaria vectors

Of the thousands of described mosquito species, only the genus *Anopheles* serve as vector of malaria parasite. There exists more than 400 different species of *Anopheles* mosquito, of which only around 40 of them serve as malaria vectors (WHO, 2019). Some anopheles species do not feed on humans, others are not susceptible to human malaria parasites, and a number of them have life spans too short to allow the parasite to fully mature. Those that are responsible for malaria transmission are abundant, long-lived, commonly feed on humans, and typically dwell in proximity to people largely depends on, the presence of a favorable environment for larval development and adult survival, and the ability to feed on humans (Ariey and Gay, 2019; Oaks *et al*., 1991). To transmit malaria, vectors must be able to support parasite development through several key stages over 8 to 15 days. Only the sporozoite stage parasites present in the salivary glands of the vector is ready for transmission to new human hosts. Of all about forty documented Anopheles mosquito species, *A. arabiensis*, *A. funestus, A. pharoensis*, and *A. nili* are the medically important malaria vectors in Ethiopia. Among these, *A. arabiensis* is a principal malaria vector largely distributed in Ethiopia (Yohannes *et al.,* 2005). The other common vectors of malaria dominating in malaria-endemic areas are *A. funestus* and *A. pharoensis* (Adugna *et al*., 2021; FMOH, 2010). *Anopheles nili* is the least common and more localized species, and it is not adequately studied. It is found in the southwestern, western, and northwestern parts of Ethiopia.

Recently, *A. stephensi* a competent malaria vector in urban environment was identified in the eastern part of Ethiopia (Balkew *et al.,* 2020).

A*nopheles* mosquitoes lay their eggs in specific moist places or aquatic habitat of their preference. The eggs hatch to produce larvae, which undergo several molts before emerging from the pupal stage as adult mosquitoes. Immediately after emerging from the pupal stage, mosquitoes rest on the water surface until their wings have fully expanded and hardened. After taking an initial meal of plant nectar, female mosquitoes seek a blood meal, as they require protein to develop their eggs (WHO, 2019). Blood feeding can take place inside human habitations (endophagy) or outdoors (exophagy), depending on the mosquito species. Several factors have been implicated in the attraction of female mosquitoes to a host, including exhaled carbon dioxide, lactic acid, host odours, warmth and moisture. Female *Anopheles* mosquitoes feed predominantly at night, although some species may bite during the day in heavily shaded conditions, and some exhibit a peak in biting activity in the early evening or early morning. The interplay between the peak biting time of the *Anopheles* vector and the activity and sleeping patterns of the human host has important consequences for malaria transmission and the choice of appropriate vector control interventions (WHO, 2019). Vector control is one of the key target in malaria control and elimination strategy, which could be achieved through advancing human economic development. Moreover, accurate species identification early control activities have a significant impact. However, the emergence and spread of insecticide resistant strains due to mutation greatly affected vector control programs (Loonen *et al.,* 2020).

2.4 Malaria control strategies and challenges

For effective malaria elimination, WHO urges affected countries and global community to maximize the impact of existing life-saving tools and strategies through ensuring universal access to malaria prevention, diagnosis and treatment, accelerating efforts towards elimination and attainment of malaria free status and transforming malaria surveillance into a core intervention (WHO, 2015). In this regards, despite significant achievements have been made so far for the control and eventual elimination of malaria; multiple challenges keep on increasing as we move ahead towards complete elimination.

Vector control is a major pillar in the fight against malaria. ITN and IRS highly contributed to the decline in malaria cases and deaths (Chukwuekezie *et al*., 2020). In this regards, Ethiopia has made

significant progresses in expanding coverage of key malaria interventions throughout the country. IRS and long-lasting insecticidal nets (LLINs) are broadly used in malaria prevention and control strategy. One major obstacle to vector control in Ethiopia and elsewhere is the ever-developing insecticide resistance mainly to the pyrethroids, due to indiscriminate and rampant use of such chemicals in public health and agricultural activities (Yared *et al.,* 2020).

Moreover, the frequent generation and spread of genetic diversity in malaria parasites mainly *P. falciparum,* ultimately circumvent not only the use of long lasting effective drugs, but also vaccine research. This is due to emergence and spread of drug resistant strains (Sonko *et al*., 2014; Mwingira *et al*., 2011). Most of the antimalarial drugs target the asexual erythrocytic stages of the parasite (blood schizonticidal drugs). There are two broad groups of such drugs. They are either fast acting (chloroquine, quinine, and mefloquine) or slow-acting (pyrimethamine, sulphonamides, and sulphone) types (Alam *et al.,* 2009). Widespread resistance to such conventional anti-malarial, motivated the WHO to recommend the use of artemisinin-based combination therapy (ACT) for the treatment of uncomplicated *P. falciparum* malaria (Plucinki *et al.,* 2015; Golenser *et al*., 2006). ACT is the cornerstone of malaria control in sub-Saharan Africa. It includes artemether/lumefantrine and artesunate/amodiaquine. The emergence of parasites resistant to artemisinins in Southeast Asia and altered sensitivities to artemisinin partner drugs pose great threats to efforts to control and, eventually, eradicate malaria (Cui *et al*., 2015; Plucinki *et al.,* 2015). Due to the notorious capacities of *P. falciparum* to develop drug resistance, many antimalarial programs have recently included dihydroartemisinin/ piperaquine (DHA/PPQ) as a second-line antimalarial drug (Plucinki *et al.,* 2015). Drug resistance limits the efficacies of many antimalarial drugs. Evidences suggests that parasites are becoming gradually resistant to the newest drugs. Molecular markers of antimalarial drug resistance could be employed to screen for the emergence and spread of such resistance and assess its spread. It provides information about the parasite genetics associated with resistance, either single nucleotide polymorphisms or gene copy number variations that are associated with decreased susceptibility of parasites to antimalarial drugs (Shibeshi *et al.,* 2020). Furthermore, the divided opinion on hyper-optimistic objective about elimination on such aggressive efforts, so that elimination may not be equally fruitful in all countries. Likewise, every country has peculiar and specific challenges on the way to elimination,

socio-cultural hindrance in malaria elimination and imported malaria cases are the additional challenges (Dhiman, 2019).

Generally, multiple challenges still exist against the available opportunities and successes in reducing malaria burden. Persistence of malaria largely in WHO African region, spread of drug resistance, behavioral adaptations of outdoor biting of the predominant malaria vectors, mass drug administration recommended in areas approaching interruption of transmission; may exacerbate level of drug resistance, resistance to insecticides and drugs-where alternatives are not ready to replace and accessing the new tools are the major ones (Ranson, 2018).

Furthermore, microscopy and RDT greatly under estimate malaria prevalence in low transmission settings coupled with the existence of hidden parasite reservoir in human host as asymptomatic cases demands molecular surveillance approach to make malaria elimination effective (D'Alessandro, 2018).

2.5 Malaria diagnosis

Prompt and accurate diagnosis is an essential component of malaria control strategies and enables the effective management of febrile patient. Malaria diagnosis involves identifying malaria parasites or antigens products in patient blood. A number of factors affect the diagnostic efficacy of malaria parasites. The existence of different malaria species, different stages of erythrocytic schizogony, could be a challenge. Moreover, the relationship between transmission intensity, population movement, parasitaemia level and, immunity affects malaria diagnosis. In addition, signs and symptoms of the disease, drug resistance strains, the problems of recurrent malaria, and sequestration of the parasites in the deeper tissues, and the use of chemoprophylaxis or even presumptive treatment based on clinical diagnosis, can all influence the identification and interpretation of malaria parasitaemia in a diagnostic tests (Tangpukdee *et al*., 2009).

2.5.1 Microscopy

Microscopy is an appropriate method for detecting and identifying malaria parasites and has been the golden standard /reference for malaria diagnosis (Nandwani *et al.,* 2003). However, it requires training and experience of microscopist. It is the primary method for morphological analysis and species identification of malaria parasite in stained blood film. Nevertheless, the protocol demands skillful and experienced microscopiest for accurate identification of species and parasitaemia level

(Comway, 2007). Thus, its reliability decreases at low-density parasitaemia. In addition, microscope cannot detect sequestered *P. falciparum* parasites. Once the diagnosis is established usually by detecting parasites in the thick smear the laboratorian can examine the thin smear to determine the malaria species and the parasitaemia, or the percentage of the patient's red blood cells that are infected with malaria parasites (CDC, 2021). Conventional light microscopy, though considered as a "gold standard" for malaria parasite identification and conformation, to achieve adequate sensitivities and specificities, it requires quality of the equipment, trained and skilled microscopists, good supervisory personnel and quality reagents (Tedla, 2019), would help as to avoid false positive and false negative results that may lead to mistreatment of the patients. Moreover, the type and quality of the smear, the parasite density, and the time spent on reading the smear are the also factors that determine the performance of microscopy. However, microscopy based diagnosis could also likely be prone to doubtful results. Likewise, it was reported that the prevalence of infection measured by microscopy was about 50.8 % of that measured by PCR and this difference could be even greatest in low transmission settings (Golassa *et al.,* 2015).

2.5.2 Rapid diagnostic tests (RDTs)

This test is based on the detection of antigens derived from malaria parasites in lysed blood, using immune-chromatographic methods. The technique most frequently employed a dipstick or test strip bearing monoclonal antibodies directed against the target parasite antigens (Moody, 2002). The technique is an alternate way of quickly establishing the diagnosis of malaria infection by detecting specific malaria antigens in a person's blood. A blood specimen collected from the patient is applied to the sample pad on the test card along with certain reagents. After 15 minutes, the presence of specific bands in the test card window indicate whether the patient is infected with *P. falciparum* or any one of the other species of human malaria parasite (CDC, 2021).

The use of RDT does not eliminate the need for malaria microscopy. All positive RDTs also should be followed by microscopy. The currently approved RDT detects two different malaria antigens; one is specific for *P. falciparum* and the other is specific to the remaining human plasmodia species, followed by microscopy confirmation (CDC, 2021). RDT test could be performed in resource-limited setting, like unavailability of electricity and a little expertise could perform the tests.

The commonly used RDT targets *P. falciparum* histidine rich protein 2 (PfHRP2) and two enzymes in the plasmodium parasites glycolytic pathways; namely plasmodial Lactate Dehydrogenase (pLDH) and Aldolase (Joel *et al.,* 2013). However, pLDH based RDT have decreased sensitivity at low Parasitaemia, because pLDH is expressed by the gametocyte. In addition, the use of antibody against Aldolase of malaria RDT is having poor performance, since falciparum Aldolase share 61-68% of sequence identity with eukaryotes aldolase (Joel *et al.,* 2013). Although it is fast, simple and less laborious in terms of quality assurance, its utility is limited in some regions because of the high frequency of parasites lacking PfHRP2 and/or PfHRP3 genes (the antigens targeted by the RDTs). However, sensitivity of this method also decreases with low parasitaemia (WHO, 2013; George, 1996).

2.5.3 Molecular methods

Molecular detection of DNA using PCR is another alternative method for malaria diagnosis. The technique involves; efficient DNA extraction, PCR based amplification and amplicon analysis for molecular diagnostic, genomic and epidemiological studies of malaria (Syaifudin, 2015). The PCR techniques, which could be applied for malaria diagnosis includes conventional PCR, nested PCR, qPCR, and multiplex PCR. Other less widely used nucleic acid-based amplification technique that do not require thermal cycler as Loop mediated isothermal amplification (LAMP) and nucleic acid sequence-based amplification (NASBA), could also be used. LAMP has been used widely to identify plasmodium species (Tedla, 2019). PCR is highly sensitive, high-throughput technology for such molecular study. Analysis of DNA by using PCR used to detect individuals with asymptomatic low-density parasitaemia, below detection limit of microscopy (~50-100 parasites/μL blood). Hence, it enables us to overcome the challenge to achieve malaria elimination (Okell *et al*., 2012). Otherwise, the case of such asymptomatic low-density parasitaemia facilitates malaria resurgence in areas where malaria had already be eliminated.

Analysis of malaria parasite DNA from the blood sample by using PCR is not only highly sensitive for plasmodia parasite detection and diagnoses, but also transforming epidemiological study of malaria. However, effective and efficient utilization of PCR demands prior knowledge of at least part of the sequence of the target DNA. This enable us to construct the specific primers to amplify the target gene (Garibyan and Avashia, 2013). The capacity of PCR to amplify extremely small amounts of DNA makes contamination a significant problem (Lynch and Brown, 1990). Taq

polymerase does not have the capacity to proof read and, prone to error, which in turn causes mutations in the PCR fragments generated. (Zhou *et al*., 1991). In addition, the size of the fragments amplified by standard Taq polymerase is limited to only a target region (Garibyan and Avashia, 2013). Moreover, other limitations of PCR includes; its protocol is not fast, needs advanced equipment, expensive reagents, managed by technical personnel only and it is not applicable in most field conditions (Mens *et al*., 2008). Understanding such PCR limitation is of great help in designing robust PCR protocol for molecular and epidemiological study.

Numerous protocols yet developed to isolate plasmodium DNA from a variety of blood sources in order to obtain high-quality DNA suitable for downstream applications. The common DNA extraction protocols are; commercial kit, Chelex- saponin/ Chelex-Tween 20®, microwave, methanol and Tris-EDTA methods (Syaifudin, 2015). DNA extraction by using commercial kit: can be utilized based on the manufacturer's protocol. The major steps of DNA extraction by using the remaining methods involves; erythrocyte lysis, washing, DNA extraction, followed by sequential purification processes. The choice of vigorous DNA extraction protocol should be simple, low cost, not labor intensive, and without compromising successful DNA extraction. If laboratory resources and facilities are not under limited setting; DNA extraction by using commercial kit method was superior to the other methods (Syaifudin, 2015). PCR has been used as a definitive method for descriminating malaria parasite species from human blood sample. First, PCR amplification of the genomic DNA extract is conducted by using genus specific primers, which amplifies a 1100bp PCR product from 18S rRNA, followed by PCR amplification by using species specific primers, forming characteristic band at 205bp for *P. falciparum* during gel electrophoresis (Johnston *et al*., 2006).

After malaria diagnosis, further characterization of the parasite through whole genome sequencing revealed, unparalleled wealth of information on plasmodium genomics. Each plasmodia genome consists of ~23 mega base (Mb) nucleotides distributed over 14 haploid chromosomes, predicted to encode about 5,400 genes, and separate mitochondrial and plastid genomes of ~6 and 35 kilobases (Kb), respectively (Sexton *et al*., 2019). The parasite chromosomes are linear and contain repetitive telomeric ends providing plasticity to the genome due to frequent deletion and insertion. *P. vivax* genome contains a much lower AT (about 55%), compared to the genome of *P. falciparum* (80%). Moreover, about 60% of the predicted genes of *P. falciparum* are encoding for hypothetical

proteins. This poses a great challenge to malaria research in identifying the vaccine targets in the genome (Gardner *et al.*, 2002).

2.6 Immunity to malaria and vaccine prospects

Acquired or adaptive immunity against plasmodia parasite includes i. anti-disease immunity, conferring protection against clinical disease, ii. Anti-parasite immunity, conferring protection against parasitaemia, and (iii) premunition, providing protection against new infections by maintaining a low-grade and generally asymptomatic parasitaemia (Doolan, *et al*., 2009). In holoendemic areas of SSA, most people are almost continuously infected by *P. falciparum*, and the majority of infected adults rarely experience overt disease (Doolan, *et al*., 2009).

Anti-parasite immune responses of the malaria parasites can efficiently control the infection at all development stages and under certain circumstances, they can prevent parasite infection (Long and Zavala*,* 2017). There are different challenges in transforming such immune response to vaccines or immune-therapeutic drugs development. The complex life cycle of the parasite with multiple stages, extensive genetic variation of the parasite particularly *P. falciparum*, and the impact of both age of infection and frequency of infection are the major ones (Rochford *et al.,* 2020; Long and Zavala*,* 2017). Immunity against malaria parasite could be innate or adaptive. The development of innate immunity could be due to epigenetic regulation of monocyte response to stimuli and the secondary response to subsequent stimuli, where repeated malaria infection occurs. Activation of Natural killer cells (NK-cells) and cytokine driven responses contributes to inflammation to antibody driven responses, whereas, adaptive immunity, involves antibodies mediating protective immunity against malaria parasite (Rochford and Kazura, 2020).

Even though, innate or adaptive immune effector mechanisms can limit the peak of parasitaemia, prevent severe pathology and reduce the load of circulating infected cells, they typically fail to eliminate the infection. This would lead to persistent low-grade parasitaemia, which might frequently fall below the limit of detection by microscopy, but which might persist for many months or years (Stevenson and Riley, 2004). Studies revealed that, older children had fewer parasites than younger children, and that frequently infected children had fewer parasites than the less frequently infected. The same report also revealed that older children were less likely to develop fever than young children and that frequently infected children were less likely to develop

fever than the less frequently infected (Stevenson and Riley, 2004, 2004). In holo-endemic area of malaria transmission, the disease symptom is confined to young children, while older children and adults are relatively protected and largely remain asymptomatic. On the other hand, in areas where malaria transmission is low, it shows unstable and seasonal pattern and symptomatic disease occurs in all age groups. The differences in the clinical consequences of malaria parasite in different transmission setting is due to differences in naturally acquired immunity (Fowkes *et al.,* 2016).

Immune responses induced by natural exposure to plasmodia has facilitated partial protection, to asexual stages of the parasite. Naturally, acquired immune responses against asexual stages are effective against parasites and that these responses reduce morbidity, even though they do not cause full parasite clearance. On the other hand, acquired immune response against the pre-erythrocyte and sexual stages shown no clear evidence of a protective immunity (Long and Zavala, 2017). The main target in analyzing such scenario is to transform the finding to a highly effective vaccine development against malaria parasite. Even though, the development of a safe, inexpensive and effective malaria vaccine is considered as one of the top global priorities for the control and eventual elimination of malaria. There are different factors that challenged malaria vaccine development. Higher genome of malaria parasite compared to bacterial and viral genome, having different stage in its life cycle, undergoing both asexual and sexual reproduction in two different hosts and having highly polymorphic surface proteins presents significant challenges for vaccine design (Bharati and Das*,* 2019; Crompton *et al*., 2010). From a number of malaria vaccine candidates, RTS, S/AS01 is presently a lead recombinant candidate vaccine against malaria (Geert *et al.,* 2014). The RTS, S is the world's first approved malaria vaccine. It is a recombinant vaccine, made by expression of the hepatitis B surface (S) antigen (HBsAg) in the yeast *Saccharomyces cerevisiae*. The S antigen is fused to the circumsporozoite protein (CSP) of *P. falciparum*, containing the repeat region (R) and a T-cell epitope (T). The vaccine is formulated with AS01, which is a liposome-based vaccine adjuvant that boosts the immunogenicity of the vaccine (, Bharati and Das*,* 2019; Zheng *et al*., 2019).

Although many promising malaria vaccines are under various stages of development, the RTS, S vaccine is the only approved malaria vaccine currently available for immunization. However, the protective immune responses after vaccination with RTS, S are dependent primarily on antibody

responses against the central repeat region. The RTS, S/AS01 vaccine, was found to protect African children against clinical and severe malaria (Karunamoorthi, 2014). It has been tested in a phase III trial and has received a positive rating from the European Medicines Agency, but it may not be effective against *P. falciparum* isolates from southern and central Africa or other global regions. The high genetic diversity in the C-terminal region of the *P. falciparum* CSP molecule may lead to the lack of overall protective efficacy. Therefore, genetic diversity assessment of the C-terminal region of *P. falciparum* CSP is an important aspect of developing an RTS, S/AS01 vaccine for widespread use (Zheng *et al*., 2019). In this regards, there are three ongoing pilot programs being implemented in Malawi, Ghana and Kenya and expected to be completed by 2023. The project envisioned that, evidence generated from these programs would enable WHO to update its malaria vaccine policy and make recommendations for the broader use of RTS, S for routine immunization (Bharati and Das, 2019).

2.7 Genetics of Plasmodium species with *P. falciparum* in focus

From all the Plasmodium species that cause human malaria, only the blood stages of *P. falciparum* is used for functional characterization of its genome. This could be through a reverse genetic approach, where a gene of interest is disrupted and the resulting phenotype is studied or through a forward genetic approach, where a phenotype of interest is first chosen and a pool of randomly generated mutants is subjected to a screen for the phenotype (Balu, 2012). A majority of the plasmodium genome remains annotated to code for hypothetical proteins with unknown functions. The introduction of forward genetics has provided novel means to gain a better understanding of gene functions and their associated phenotypes. The technique has already shown significant promise to increase our understanding of parasite biology needed for rationalized drug and vaccine design (Balu, 2012).

Malaria parasite genomes carry multigene families that serve important roles in parasite interactions with their hosts, including antigenic variation, signaling, protein trafficking, and adhesion. Among the gene families, the genes encoding *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) have been studied most extensively. Each individual *P. falciparum* parasite carries a unique set of about 60 copies of the var genes in its genome, where switches of gene expression produce antigenic variation (Su, 2019). PfEMP1 plays an important role in the

pathogenesis of clinical developments such as in cerebral and placental malaria, in which it mediates the cyto adherence of infected red blood cells (iRBCs; infected erythrocytes) in the deep tissue. Different PfEMP1 molecules bind to various host molecules, such binding leads to activation of various host inflammatory responses. This poor display of PfEMP1 on the host cell surface offers protection against malaria by reducing the cyto-adherence and activation of inflammatory processes that promote the development of severe disease (Su, 2019. Cyrklaff et al., 2016).

Malaria parasites devote large portion of their genomes to gene families that ensure evasion of host immune defenses and protection of molecular processes essential to infection. These families emphasize the importance of research on their roles in parasite-host interactions and virulence, despite the difficulties inherent to their investigation (Su, 2019). There are also proteins often localized on the parasite surface interacting with host proteins. The proteins expressed in asexual stages of the parasite development are generally polymorphic and/or under selection, suggesting that they could be targets of the host immune response; undergoing various selection pressures due to host immunity, thus targeted for vaccine research (Williamson, 2003). Similarly, the genes that expressed at different parasite developmental stages are; pf230, pf48/45, pf230p, pf47, and PfPSOP12 expressed in *P. falciparum* sexual stages; pf52, pf36, pfLISP2, and pfB9 are expressed in the pre-erythrocytic stages; and pf12, pf12p, pf41, pf38, and pf92 are expressed in asexual erythrocytic stages (Su, 2019, Arredondo and Kappe, 2017). Furthermore, *P. falciparum* genome is largely conserved in central regions but extensively polymorphic in both length and sequence near the telomeres, and much of this sub-telomeric variation was explained by recombination within blocks of repetitive sequences and families of genes (Freitas-Junior et al., 2000).

In *P. falciparum* there is approximately one polymorphic microsatellite per kb DNA, a high rate that may involve the AT-rich nature of the genome. This abundance of microsatellites facilitated the generation of high-density genetic linkage maps (Su *et al.,* 2007), that could be expedited for PCR-based mapping of specific locus for parasite characterization and molecular epidemiological study. Furthermore, advances in DNA sequencing technologies have also facilitated the identification of large numbers of single nucleotide polymorphisms (SNPs). High-density SNP maps for the parasite could be used for genomic and epidemiological study of the parasite populations. Another important type of genomic variation in Plasmodium is copy number variation

(CNV). CNVs have been found to affect important traits of drug resistance, erythrocyte invasion, cytoadherence, and transcriptional regulation (Su, 2019).

With rapid development of sequencing technologies, a large number of *P. falciparum* isolates worldwide have been sequenced and have generated the parasite genomic data that has generated numerous high-density single-nucleotide polymorphisms (SNPs) of the parasite. Application of such genomic approaches could enable us to identified new parasite genes that conferred the resistance to antimalarial drugs (Amato *et al.*, 2016)

Sequence-based GWA studies are powerful tools for phenotypic association tests with novel SNPs while broadly surveying known polymorphisms (Van Tyne et al., 2011). GWASs approach provide guidance to future research directions like: the genetic architecture of malaria susceptibility/resistance, heritability of malaria susceptibility and its distribution across the genome at genome wide scale (Damena *et al*., 2019).

Taken together, knowledge of genetic variations and genome diversity of malaria parasite populations has greatly improved our understanding of the biology, gene function, drug resistance, population dynamics, transmission, and molecular evolution of malaria parasites (Su, 2019).

2.8 Genetic diversity of *P. falciparum*

As described elsewhere, *P. falciparum* is the most prevalent and the most virulent malaria parasite and responsible for over 90% of cases and death mainly in SSA. *P. falciparum* is most affected by resistance to antimalarial drugs, which constitutes a major challenge in the fight against malaria. For instance, in Ethiopia, a high prevalence of *P. falciparum*, mutant alleles detected in codons related to chloroquine (CQ), chloroquine resistance transporter (p*fcrt)* and multidrug resistance protein 1 (*pfmdr*1) and sulfadoxine- pyrimethamine (SP) dihydropteroate synthase (*pfdhps) genes* and falciparum dihydrofolate reductase (*pfdhfr*) resistance. Thus, CQ and SP was withdrawn due to a high prevalence of CQ and SP resistance in *P. falciparum* (Lo *et al*., 2017). It was the emergence and spread of such drug resistance that necessitated WHO to introduce ACT, as the first line drug for the treatment of uncomplicated *P. falciparum* malaria. This consequently resulted in substantial reduction of the global burden of the disease in all malaria endemic regions. Although ACTs are still generally accepted as the best treatments for uncomplicated falciparum malaria, reports confirmed that resistance to these drugs emerged in Asia and South America and

spreading to Africa (Nosten and White, 2007), which consequently could worsen the situation, demanding proactive responses.

The driving force for the emergence and re-emergence of drug resistant strains in *P. falciparum* is the genetic diversity within the parasite species. People living in malaria endemic area frequently and simultaneously get infected by several strains of the parasite (Sondo *et al*., 2019). Understanding the genetic diversity of malaria parasites from different regions is important to analyze the genetic profile of the parasite population dynamics and transmission intensity. Furthermore, it is also valuable in discriminating parasite clones from infected individuals and tracing the origin of parasites (Soe *et al.*, 2017). Genetic diversity is an indicator of the evolutionary fitness of a parasite population as high genetic diversity provides greater potential for adaptation to changing environmental conditions and for immune escape, due to antigenic polymorphisms (Takala *et al*., 2010). The genetic diversity of the parasite is one of the main factors responsible for the slow acquisition of immunity against malaria. Thus, individuals must meet the entire diversity of circulating parasite populations before they develop an effective anti- malarial immunity (Sondo *et al*., 2019). Similarly, genetic diversity of malaria parasites would enhance their ability to counteract the therapeutic drugs. Moreover, it is also one of the major barriers to the development of malaria vaccines. In hyper-endemic areas, each individual host may harbor multiple malaria parasite strains with different genotypes, including those that confer drug resistance (Robinson *et al*., 2011). Sexual reproduction involving malaria parasites of different genotype could occur when a mosquito feeds on an individual infected with multiple parasite strains or when the same mosquito feeds on more than one human bearing distinct parasite genotypes. This favors genetic recombination and generation of higher diversity that tends to correlate with the transmission intensity (Soe *et al.*, 2017). On the other hand, studies have shown that malaria reduction as the result of intensified control efforts result in reduced genetic diversity of the parasite populations (Park *et al.*, 2013). Extensive genetic diversity in natural population is a major obstacle for the development of effective vaccine against the human malaria parasite, since antigenic diversity limits the efficacy of acquired protective immunity to malaria (Recker *et al*., 2011). Extensive use of certain antimalarial drugs can also alter the genetic diversity due to selective sweeps. Genetic diversity, malaria demographic history and other factors in a given region may reflect the transmission intensity, effectiveness of malaria control measures, and

potential emergence of resistant parasites. Therefore, a better understanding of *P. falciparum* antigenic diversity in a given locality is paramount to plan for effective malaria intervention.

Different approaches in molecular techniques could be used to analyze genetic diversity of falciparum populations for epidemiological and drug efficacy purposes within countries and across continents. Of these microsatellite loci analyses have been suggested due to their abundance, putative neutrality and higher levels of polymorphisms with tandem repeats of 2–6 bp (Ajogbasile *et al*., 2021). Microsatellites are widely distributed throughout the genome. In *P. falciparum*, there are 12 microsatellite markers that could be readily amplified by PCR, followed by gel-electrophoresis. The inability of agarose gel electrophoresis in discriminating of alleles of similar sizes and allele size difference less than 20 bp, resulting in underestimates of diversity and multiplicity. However, the use of capillary electrophoresis based fragment analysis could minimize such limitations (Zhong *et al*., 2018).

The genome of *P. falciparum* could be also be characterized by substitutions of single nucleotides occurring at specific positions in the genome, referred to as single nucleotide polymorphisms (SNPs). SNPs are considered useful molecular markers for barcoding to identify and characterize genetic diversity of *P. falciparum* genotyping. SNPs and indels analysis employed through next generation sequencing (NGS) could show more allelic families than identified by PCR method (Metoh *et al.,* 2020). Besides, SNPs are ideal genetic markers for tracing the origin and migration of the parasites. Analysis of the whole genome variation has also generated numerous high-density SNPs of the parasite. Application such genomic approaches has identified new parasite genes that conferred the resistance to ACT antimalarial drugs (Amato *et al.,* 2016). SNPs could be studied through, amplicon ultra-deep sequencing and whole genome sequencing techniques with high level of sensitivity and specificity. However, the costs and time factors attached to the use of these techniques are relatively high, thereby limiting their application especially in resource-limited settings (Bosco *et al.,* 2021).

In such cases, the genetic diversity of *P. falciparum* parasites can be studied by using polymorphic markers like: Merozoite Surface Proteins MSP-1 and MSP-2 (Soe *et al*., 2017). Molecular epidemiological study through genotyping of such polymorphic marker is the decisive approach to determine the type and clones of the parasite infection. In this regard, PCR amplification followed by gel or capillary electrophoresis is the most widely used technique for malaria parasite

genotyping of the polymorphic genes mainly *msp*-1 and *msp*-2 (Bakhiet *et al.,* 2015*,* Ogouyemi *et al.,* 2013).

2.8.1 *Plasmodium falciparum msp*-1 and *msp*-2 genes

The interaction of parasite merozoite surface proteins with erythrocyte mediate its invasion. The nature of these interactions is currently not well understood, but as depicted in (Figure. 1), it is known that MSP-1 is critical for successful erythrocyte invasion (Lin *et al*., 2016). MSP-*1* and MSP-*2* proteins are two major *P. falciparum* blood-stage malaria vaccine targets *(*Chitarra *et al.,* 1999). They are involved in erythrocyte invasion (Nilsson *et al.*, 2018; Holder, 2009). Both being targeted by the immune responses (Woehlbier *et al.*, 2006). MSP-1 is a 190 KDa surface protein encoded by the *msp-1* gene located on chromosome 9 and contains 17 blocks of sequences flanked by conserved regions (Hamid *et al*., 2013, Snounou and Singh, 2002, Smythe *et al.*, 1991).

Synthesis of MSP-1 starts during the onset of schizogony as a precursor that rapidly associates with MSP-7 and the complex transported to the surface of the intracellular parasite where it retained because of its glycosylphosphatidyl inositol (GPI) anchor. At the end of schizogony, merozoite release (or egress) from the infected red blood cell is accompanied by proteolytic processing of the complex (Holder, 2009). The potential of the molecule as a vaccine candidate demands understanding of the structural diversity of the MSPs and its immunogenicity and antigenicity. Many *P. falciparum* proteins, including MSPs modified extensively during schizogony to ensure successful invasion of the merozoite into human erythrocytes. However, the peripheral merozoite surface proteins do not have a transmembrane domain or GPI anchor and therefore interact with anchored proteins presented on the merozoite surface (Lin *et al*., 2016).

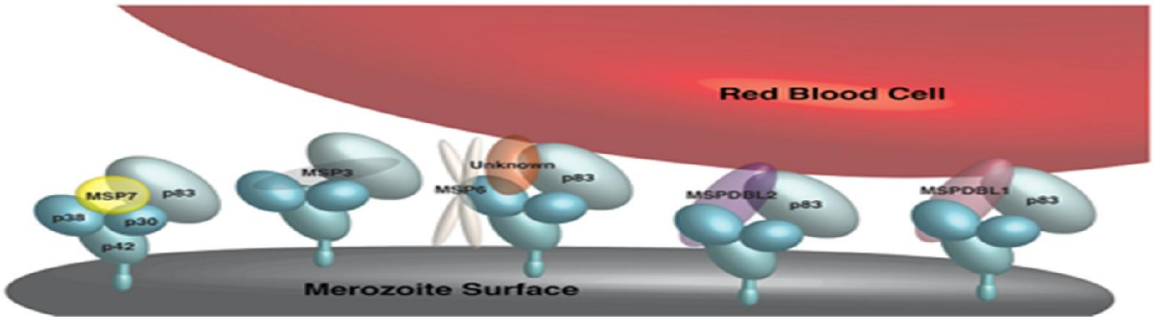


FIGURE 2: The binding of MSP complex with erythrocyte during its invasion.

The life cycle of malaria parasite commences with the merozoite making contact with the erythrocyte through its anterior apex. In a complex series of coordinated events the apical organelles (micronemes and rhoptries), release their contents. Moreover, it connects the parasite and erythrocyte surface, and then the merozoite moves into the erythrocyte to reside inside the parasitophorous vacuole (PV). Within the PV, the parasite develops from ring to trophozoite stage and then undergoes nuclear division forming schizont stage to form new merozoites that are released at the end of the cycle by rupture of the red blood cell (RBC) (Kadekoppala and Holder, 2010). The peripheral merozoite surface proteins MSP3, MSP6, MSPDBL1, MSPDBL2, and MSP7 bind directly to MSP-1, but independently of each other, to form multiple forms of the MSP1 complex on the parasite surface (Lin *et al*., 2016).

The precise functional role of MSP-1 during invasion has not yet been fully evaluated, and its macromolecular characterization have been incomplete (Lin *et al*., 2016). However, the characterization of *msp-1* gene is playing important role in identification of genetically distinct *P. falciparum* strains, which is useful to investigate genetic diversity, multiplicity of infections (MOI) and parasite carriage. Polymorphism in *msp-1* and *msp-2* genes have been frequently reported from different parts of the world. Of the 17 blocks of *msp*-1, block 2 is the most polymorphic region characterized into three allelic families namely (K1, MDA20 and RO33) *(*Cowan *et al.*, 2014, Snounou and Singh, 2002), as shown in (Figure. 2). Based on variation in length and sequence diversity, this region is a commonly targeted part in determining genetic diversity and MOI in clinical isolates of *P. falciparum* (Mohammed *et al.,* 2015, Snounou and Singh, 2002).

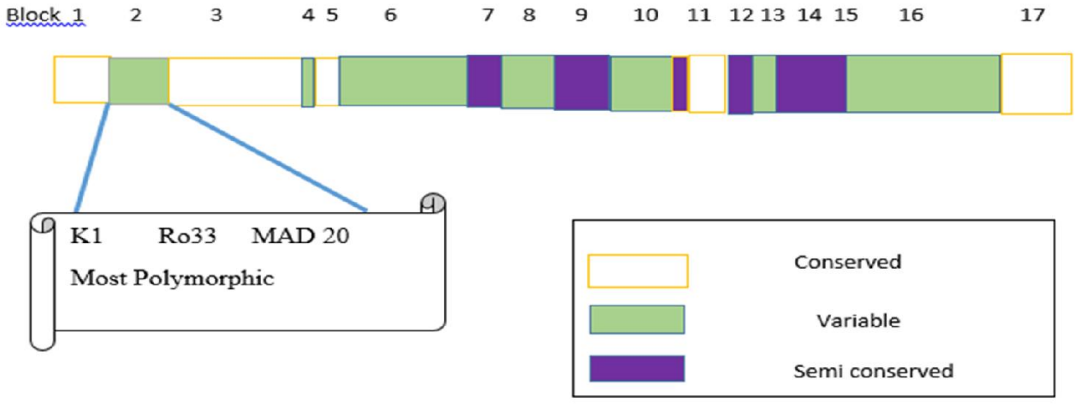


FIGURE 3: Schematic diagram of the *msp*-1 gene of *P. falciparum* (K1, RO33, and MAD 20) sub allelic regions.

Highly polymorphic *msp*-2 antigenic marker is used extensively to describe the parasite populations, thus used as a discriminatory and informative marker for strain differentiation (Mwingira *et al.*, 2011). Due to the variable, non-repeat sequences as well as the varying sizes of the tandem repeats in the central region, *msp*-2 gene is a dimorphic and existing in two main allelic families; FC27 and ICI/3D7, as depicted in (Figure.3), (Duah *et al*., 2016; Snounou and Singh, 2002). The naming is derived from the parasite lines from which they were first observed (Maier *et al*., 2009; Snounou and Singh, 2002). Based on variation in length and sequence in central region *msp*-2 gene have been used to determine MOI in clinical isolates of *P. falciparum.* In FC27 allelic family there are three blocks similarily 3D7 allelic family also sub classified into three blocks. In FC 27 liked family the amino acid length of each block is indicated. In both sub allelic families Block 3 consists of two different repeat units (R1 and R2), which are separated by non repeat region (NR). In 3D7, R1 had Glycine (G), Serine (S), and Alanine (A) enriched sequence, while R2 featured Threonine (T) repeats, whereas in FC27, R1 & R2 had (32aa)n and (12aa)n respectively (Chaorattanakawee *et al.*, 2018)

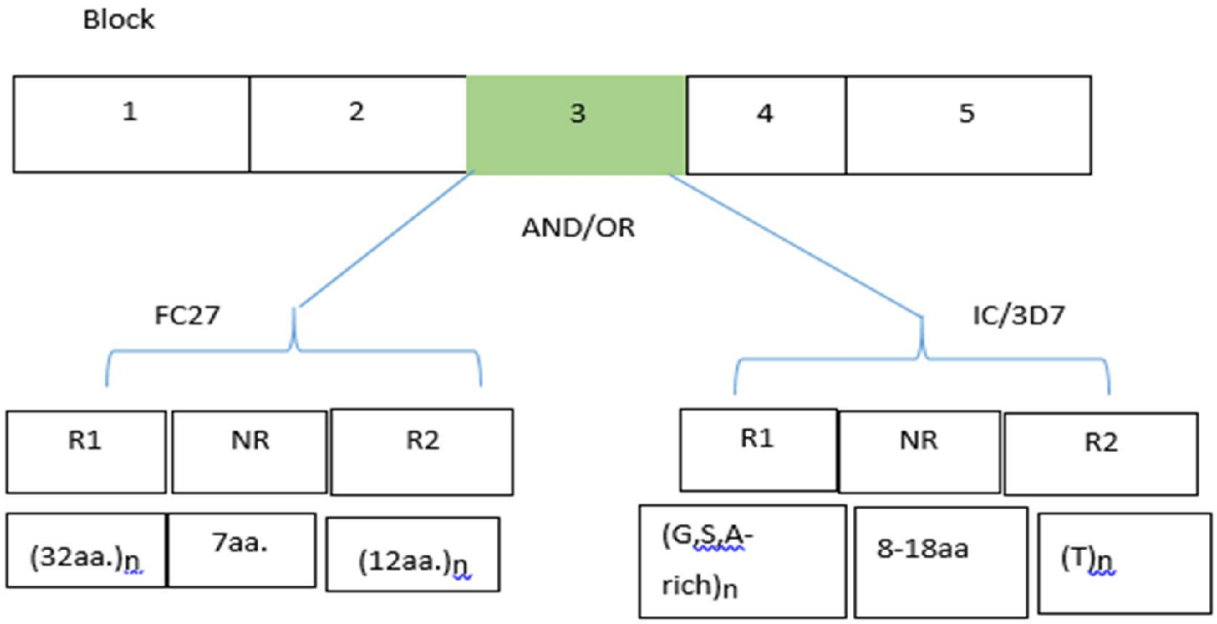


FIGURE 4: Schematic diagram of the *msp-2* construct of *P. falciparum* (3D7 and FC27) sub allelic regions.

MSP-2 is involved in RBC invasion, because anti- MSP-2 antibodies like synthetic MSP-2 peptides shown to inhibit merozoite invasion and parasite growth (Richards and Beeson, 2009). Moreover, *msp-2* gene is a target of naturally acquired clinical immunity to malaria. *msp*-2 gene

is exceptionally interesting as a candidate marker for parasite virulence given its pathogenicity and genetic diversity, with a high degree of both length and sequence polymorphism (Chaorattanakawee *et al.*, 2018).

One of the major barriers to the development of malaria vaccines is the existence and frequent emergence and spread of genetic diversity of the leading vaccine candidate antigen in malaria parasite in general and *P. falciparum* in particular. The maintenance of such variants in the population implies that they have a selective advantage like evasion of pre-existing immune responses. Alternatively, the mutations might be selectively neutral, thus population diversification could occur randomly.

Genetic diversity may arise due to multiple point mutations or variations in the numbers, lengths, and sequences of amino acid repeats. Repeat diversity could arise from intragenic recombination, misalignment of repeated DNA sequences or complementary-strand slippage during DNA replication (Franks *et al*., 2003). The AT-rich nature of the *P. falciparum* genome may also facilitate the generation of new variants (Rich *et al*., 2000), due to higher rate of indel mutation. Therefore, although the development of effective malaria vaccine is urgently needed for malaria control and its elimination; extensive genetic diversity in natural malaria parasite population is a major obstacle (Takala *et al*., 2010).

In endemic areas, malaria transmission is not consistently stable; it dependent on factors such as climate, the location of mosquito breeding sites, human related factors like nature of habitations that could serve as parasite reservoirs for effective transmission (Laishram, *et al*., 2012). Sexual reproduction involving malaria parasites of different genotypes favors genetic recombination and generation of higher diversity (Soe *et al.*, 2017). Study of the genetic diversity by using surface proteins like MSP-1 and MSP-2 is informative on the transmission intensity, how the ongoing control measures are translated in to effective interventions, and the potential emergence of drug resistant strains against therapeutic drugs (Soe *et al.*, 2017).

Msp1 and *msp2* are blood stage *P. falciparum* surface antigens directly accessible to host immune effectors targeted by the immune responses (Woehlbier *et al*., 2006), such as antibodies, complement, neutrophils, or monocytes (Joos *et al*., 2015). Antibodies against various recombinant MSPs have been associated with protection against clinical episodes of *P.*

falciparum malaria in endemic settings (Joos *et al*., 2015). However, extensive polymorphisms of such surface antigens contribute to the immune evasion, emergence of drug resistant variant forms and appear to restrict the effectiveness of vaccine against the *P. falciparum* polymorphic proteins (Patel *et al*., 2017).

2.8.2 Multiplicity of *P. falciparum* infection

MOI also termed complexity of infection (COI) refers to number of genetically distinct plasmodium parasite types simultaneously infecting a patient. It may be generated by multiple bites with infected mosquitoes or by a single bite from a mosquito infected with multiple genetic parasite types, which is an important indicator of malaria epidemiology (Hamid *et al*., 2016; Galinsky *et al*., 2015; Kyabayinze *et al*., 2008).

The use of PCR amplification is to estimate prevalence and incidence of the parasite that include subclinical cases of parasitaemia. It helps to assess the effectiveness of intervention strategies on the (occurrence, complexity, and duration of infections). In addition, it helps us to differentiate recrudescence from relapse, and new infections. It also enable us to estimate the effect of interventions on the allele frequency of the targeted gene, estimation of the differential contributions of individual hosts to the transmission by targeting gametocyte-specific genes, assessment of demographic patterns within parasite populations (gene flow, migration, colonization of new areas and population expansions) (Syaifudin, 2015). Application of such molecular techniques have shown not only mixed infections with two or three species of parasite (Bousema *et al*., 2010, Maitland and Marsh, 2004), but also mixed strain infections or MOI of *P. falciparum*. MOI could be the result of two different processes, the co-transmission of different parasite variants (co-infections) or the overlap of genetic variants due to infectious contacts before the primary infection is resolved (superinfections) (Pacheco *et al.*, 2016). Different molecular epidemiological studies in malarious areas have shown that many individuals co-infected with several different clones of plasmodia species endemic to that locality. MOI varies depending on the overall prevalence of infection in the population and the age of the individual (Soulama *et al.*, 2009), and it may be predictive of the individual's subsequent risk of clinical malaria (Pinkevych *et al*., 2014). MOI can be an indicator of the status of immunity.

Higher endemic settings are prone to infections containing multiple *P. falciparum* isolates, primarily due to repeated exposure to mosquitoes infected with multiple parasite isolates (Das *et al.,* 2017). Genetically diverse parasites can result in persistent infections, as some parasite isolates may be resistant to the drug used for treatment and thus remain after the treatment (Amoah *et al.,* 2017), spreading the transmission of resistant strain and the concomitant disease severity.

PCR based analysis on MOI of samples from highly endemic *P. falciparum* has shown that, infections with multiple parasite clones are common in high transmission settings (Karl *et al.*, 2016), and limited diversity has been found in some areas of low transmission (Nabet *et al*., 2016). On the contrary, there are also reports that, proportions of MOI remain relatively high despite seemingly low transmission (Van Den Eede *et al*., 2010). One of the possible reasons for this scenario is that, individuals residing within local transmission hotspots may be subject to much higher rates of infectious bites, therefore maintaining higher MOI levels even at very low overall parasite prevalence in the larger population (Perkins *et al.,* 2013). Such multiple-strain plasmodia infections are believed to play an important role in the development of strain-specific immunity (Kyabayinze *et al*., 2008), and MOI increase opportunities for the uptake of genetically distinct clones by the mosquito vector and for the generation of genetic diversity through meiotic recombination (Barry *et al*., 2015), resulting the transmission of multiple strains.

Understanding of the status of MOI of *P. falciparum* in a given locality, its relation with clinical severity and parasitaemia level in a population is crucial in molecular epidemiological evidence for targeted intervention. The seasonal variation of MOI with age group and effect of erythrocyte variant and MOI and the like enlighten our understanding of the local epidemiology of malaria and the assessment of the effectiveness of operational control and elimination efforts in a given setting. MOI can also be an indicator of the status of immunity. As immunity develops, MOI seems to increase. On the other hand, other studies have shown an inverse association (Pinkevych *et al*., 2014). Thus, the relationship between the level of immunity with MOI plasmodia species, and the level of MOI and the transmission intensity, seasonal and spatial dynamics need further investigation.

To sum up whole genome sequencing of *P. falciparum* helps to identify genetic diversity and MOI and estimate maximum number of haplotypes in specific genome, through extensive and efficient use of computational algorithms and mathematical models. However, the protocol is costly to

generate the expected large-scale data (Zhong *et al.,* 2018). On the other hand, PCR-based genotyping methods using *msp-1*, *msp-2,* and microsatellite markers often underestimate MOI. This problem could be more pronounced when using microsatellites (Oralee *et al.*, 2020; Zhong *et al*., 2018). However, characterization of PCR amplicon of the microsatellite markers by capillary electrophoresis gives better estimate of genetic diversity and MOI (Zhong *et al.,* 2018). Due to the aforementioned challenges, in resource limited setting genetic diversity and MOI can be studied through PCR amplification and agarose gel electrophoresis following prior protocol optimization, stringent protocol formulation and PCR programing, to minimize such limitations.

3. MATERIALS AND METHODS

3.1 Methodological and conceptual framework

The study was conducted following structured sequential steps for data collection and processing designed after reviewing relevant literatures and existing Molecular Biology protocols, as summarized in (Fig. 5) below.

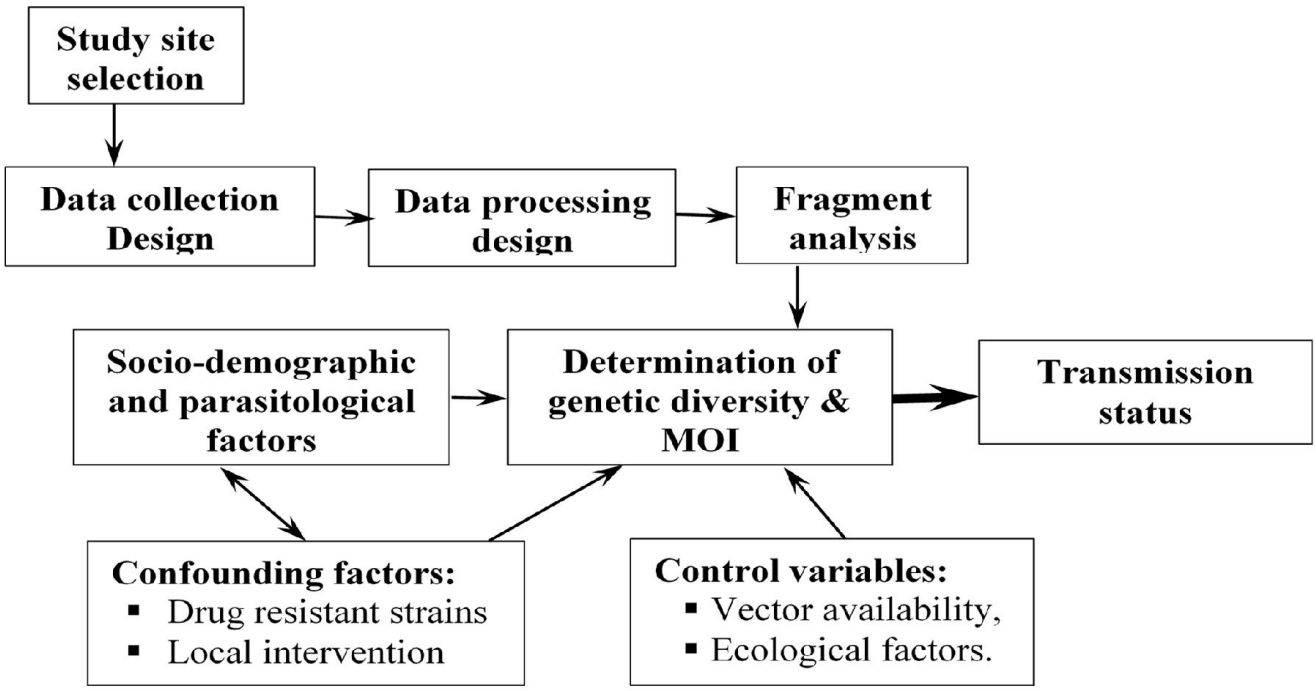


FIGURE 5**:** Methodological and conceptual framework of the study.

3.2 Study design and sample sites

The study was health facility based cross sectional study on malaria cases due to *P. falciparum* identified by microscopy protocol. The collection of blood sample and relevant patient data was carried out from September 2019 to August 2020. The sites included in the study were mainly from Adama town. The remaining are from; Adama district including Wonji located at 8km south of Adama town and Awash Malkasa situated at a distance of 15km southeast of Adama town and other health facilities located in neighboring districts like Modjo located at 16 km northwest of Adama and Olanciti situated at 23km northeast of Adama (Figure.6). Adama is the major town next to the main capital in central Ethiopia, located at a distance of about 84km southeast of Addis

Ababa through the express highway. Similar to the other part of the country where malaria is endemic, malaria transmission in Adama and its surroundings is seasonal based on the rainfall patterns that is heavy from mid -June to mid-September which accounts for major malaria transmission season from mid-September to December. And shorter rainy period near April resulting minor malaria transmission until June (Golassa and White, 2017). According to the 2007 projection of population census, catchment population of the study area estimated to reach 800,000 inhabitants. Adama and its neighboring districts are located in the Great Rift Valley area of central Ethiopia, where malaria is endemic. The major factors contributed for such malaria endemicity are: topographic location 1436 -1850m above sea level, seasonality of rainfall pattern, average annual temperature from 16–32°C suitable for the breeding of *Anopheles gambiae* (the predominant malaria vector in the region), and various micro ecological factors that favor mosquito breeding (File *et al.,* 2019, Golassa and White, 2017).

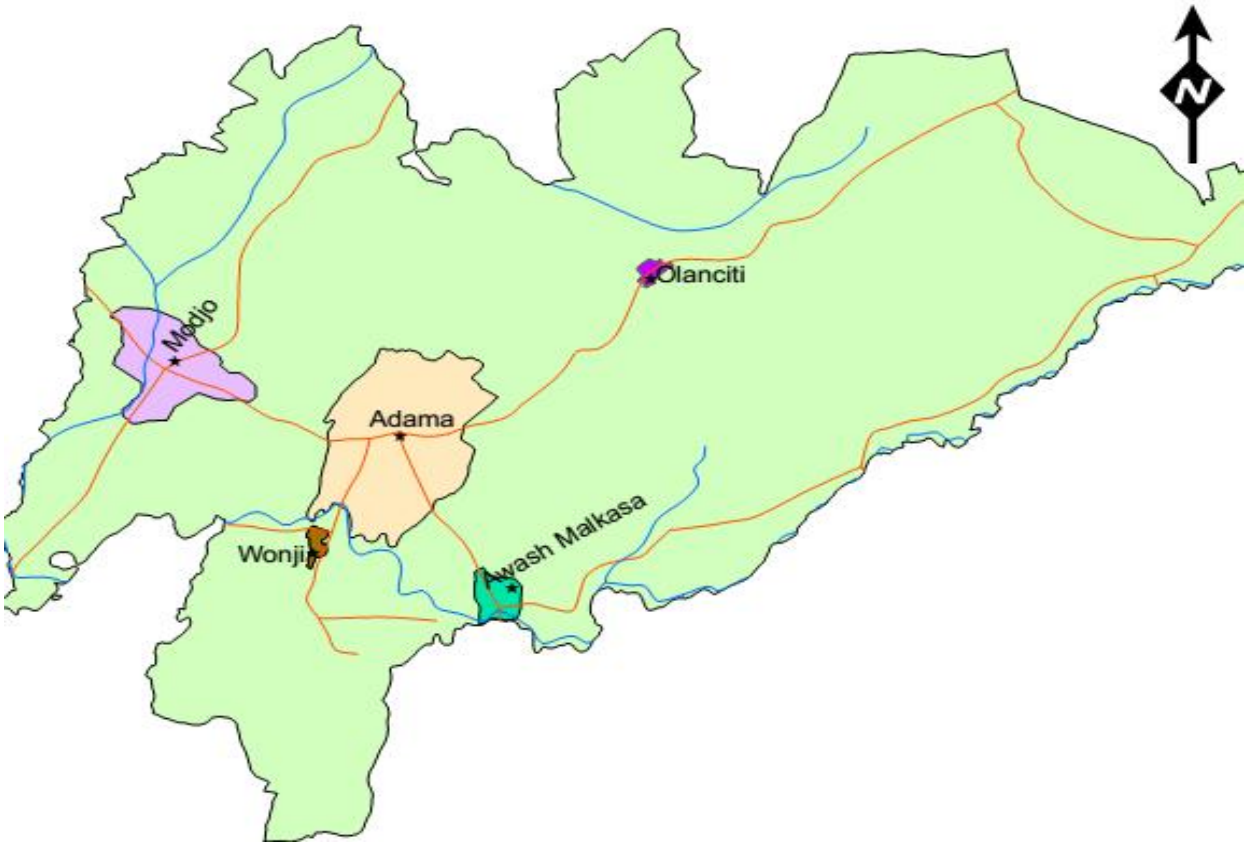


FIGURE 6: Map of the study area.

(Figure developed by using Arc-GIS Desktop version 10.4).

3.3 Study design, blood sample collection and processing

Blood sample from one hundred seventy one (171) microscopically confirmed *P. falciparum* infected patient were collected including samples from the major and minor malaria season in the study area following (SOPs: appendices 8.6, 8.7, 8.8 and 8.9). The age of malaria patients enrolled in the study ranged from one to sixty-six years. The sampling procedure involves convenient sampling technique to collect a minimum of 150 samples from purposively selected five health facilities in Adama town and four from the nearby small towns. The inclusion criteria for this study were uncomplicated malaria cases due to *P. falciparum,* having history of fever onset since 24 hours of the clinical examination. In addition, patients with complicated malaria cases, due to *P. falciparum* and patients who refuse to give consent were excluded from the study. Health facilities in these cross sectional study sites were selected based on physical location and history of patient caseload. For the sample and patient data collection, qualified and experienced medical laboratory technologists were recruited from each health facilities. The recruited laboratory professionals trained to conduct finger prick to collect blood sample for Dry Blood Spot (DBS) preparation, drying, and temporary storage together with respective patient data. For malaria microscopy, thick and thin blood films prepared, air-dried and stained with 10% Giemsa for 15 minutes. The identified Falciparum positive microscopic slides were collected from sample collection sites for further analysis. Two independent WHO certified laboratory technologists for malaria microscopy proofread the slides for malaria parasite species identification and parasitaemia level, at Adama malaria diagnostic center. Parasite density was calculated by averaging the two closest counts.

The number of parasite density per microliter (µl) of blood was calculated by using the following formula (Hamid *et al*., 2016, WHO, 2016):

$$\text{Parasite density}/\mu\text{l} = \frac{\left(\begin{matrix}\text{Number of asexual}\\ \text{parasite per 200WBC}\end{matrix}\right) \times (\text{Assumed WBC}/\mu\text{l})}{200}. \quad (1)$$

Assuming that "X" number of asexual parasite counted per 200 WBC in thick film, and about 8000 WBC expected to be present in 1µl of blood.

For comparison with ranked order variables, parasitaemia were categorized into: (50-499 parasite/µl blood), (500-4999 parasite/µl blood), (5000-49999 parasite/µl blood), and (≥50,000

parasite/µl blood) (Diouf *et al*., 2019). *P. falciparum* positive blood was spotted on Whatman ™ 3MM filter paper, air dried, and singly placed in a plastic bag with desiccant and later transported to Adama public health referral and research laboratory center for storage in deep freeze (-20 degree Celsius).

3.4 Extraction of *P. falciparum* DNA

Parasite genomic DNA extraction and its genotyping were conducted at malaria research laboratory, Aklilu Lemma Institute of Pathobiology, Addis Ababa University (AAU). The extraction of genomic DNA of *P. falciparum* from the spotted Whatman 3MM filter paper was carried out by using 0.5% Tween® 20 (Sigma-Aldrich, USA) to lyse RBC, tracked by treatment with 6% chelex ® 100 (Sigma-Aldrich, USA) in water bath at 96 °C (Snounou and Singh, 2002) following appendix 8.1.

3.4.1 PCR amplification of *msp-1* and *msp-2* genes

The genus and species-specific nested amplification was carried out targeting 18s rRNA gene as described by Snounou and Singh (2002). The polymorphic regions of *P. falciparum msp-1* gene and *msp-2* gene were used as a genetic marker for the genotyping of parasite populations. Nested PCR of *msp-1* (block 2) and *msp-2* (block-3) polymorphic region was performed by slight modifications of the primers and methods from previously described works (Chen *et al.*, 2018; Farooq *et al*., 2009; Snounou and Singh, 2002) as summarized in (Table 1). In brief, initial amplification (N1) of *msp*-1 and *msp*-2 gene were carried out in a final volume of 20µl amplification mixture containing 1x FIREPol® Master Mix (4µl), 0.5 µl of each primer (M1-OF/M2-OF and M1-OR/M2-OR), 11 µl of nuclease free water aliquot to 16µl to which 4µl of DNA template was added. In the second (N2) amplification reaction, 20µl amplification mixture containing 1x FIREPol® Master Mix (4µl), 0.5 µl of each primer for the respective amplification *msp-1* (MAD20, K-1 and RO33) and *msp-2* (3D7 and FC27) allelic variants, 13 µl of nuclease free water aliquot to 18µl to which 2µl of DNA template was added.

The PCR amplification profile for both N1 and N2 reactions includes; Initial Denaturation at 95°C at 3 minutes, Denaturation at 94°C for 1 minute, annealing 58°C for 1 minute, elongation 72°C for 2 minutes and final elongation at 5minutes (appendices 8.2, 8.3, and 8.4). Allele specific positive control 3D7 and DNA free negative control were used in each set of the reactions.

Table 1: Primers used in the study to amplify outer region (N-1), and inner allelic families (N-2) of *msp*-1 and *msp*-2 allelic families.

Amplification	Primer name	Sequence	Target gene/allele
Plasmodium genus specific	rPLU6	5'- TTAAAATTGTTGCAGTTAAAACG-3'	18s rRNA
	rPLU5	5'-C**Y**T GTT GTT GCC TTA AAC TTC-3'	
P. falciparum spp. specific	rFAL1	5'-TTA AAC TGG TTT GGG AAA ACC AAA TAT ATT-3'	
	rFAL2	5'-ACA CAA TAG ACT CAA TCA TGA CTA CCC GTC-3'	
msp-1 gene	M1-OF	5'-CTA GAA GCT TTA GAA GAT GCA GTA TTG-3'	*msp*-1
	M1-OR	5' CTT AAA TAG TAT TCT AAT TCA AGT GGA-3'	
msp-1 allelic families	M1-KF	5'- AAT GAA GAA GAA ATT ACT ACA AAA GGT-3'	K-1 allele
	M1-KR	5'- GCT TGC ATC AGC TGG AGG GCT TGC ACC-3'	
	M1-MF	5'- AAA TGA AGG AAC AAG TGG AAC AGC TGT-3'	MAD20 allele
	M1-MR	5'-ATC TGA AGG ATT TGT ACG TCT TGA AT-3'	
	M1-RF	5-TAA AGG ATG GAG CAA ATA CTC AAG TTG TTG-3'	RO33 allele
	M1-RR	5'-CAT CTG AAGGAT TTG CAG CACCTG GAG-3'	
msp-2 gene	M2 - OF	5'-ATG AAG GTA ATT AAA ACA TTG TC TA-3'	*msp*-2
	M2 - OR	5'- CTT TGT TAC CAT CGG TACA TT CTT-3'	
msp-2 allelic families	FC27 (B1)	5'-GCA AATGAA GGT TCT AAT ACT AAT AG-3'	FC27 allele
	FC27 (B2	5'- GCTTTGGGTCCTTCTTCAGTTGATTC-3'	
	IC/3D7 (A1)	5'-GCA GAA AGT AAG CCT TCT ACT GGT GCT-3'	IC/3D7 allele
	IC/3D7 (A2)	5'- GATTTGTTTCGGCATTATTATGA-3'	

3.4.2 Detection of *msp-1* and *msp-2* fragments by gel electrophoresis

Following the developed SOP (appendix 8.5), gel-electrophoresis of DNA fragment for *msp-1* and *msp-2* allelic families were performed on 2% agarose gel stained with ethidium bromide and visualized under Benchtop 2UV trans-illuminator (UVP), USA and photographed to estimate band size in relation to 50bp DNA ladder (Invitrogen, by thermal Fisher-scientific). The detection of one PCR fragment on each locus indicates infection is monoclonal, whereas the presence of more than one fragment on each locus shows polyclonal infection (Kiwuwa *et al.*, 2013). If the size of allele fragment is less than 20 bp interval they are considered the same (Ogouyèmi-Hounto *et al.*, 2013) as shown in appendix 8.6A and 8.6B.

3.5 Data Analysis

MOI is the number of distinct parasite genotypes co-existing within the given infection, which could be estimated by dividing the total number of gel fragments detected in respective gene by the total number of positive samples in the same marker that indicates the average number of genotypes per infected subject. The mean MOI can be calculated by dividing total gel fragments by the total number of positive sample for *msp-1* and *msp-2* genes. Allele frequency represents the incidence of allelic variant in the sample isolates. It could be determined by dividing the number of times the allele of interest is observed in a population by the total number of copies of all alleles detected for *msp*-1 and *msp*-2 gene in the isolates.

Descriptive statistics was used to calculate the frequency of each *msp-1* and *msp-2* allelic families in relation to the total number of gene successfully amplified for the specific locus. MOI for *P. falciparum* calculated as total number of parasite genotypes for the same gene and the number of PCR positive isolates. Size polymorphism in each allelic family shows that one band represents one amplified PCR fragment derived from a single copy of *P. falciparum msp*-1 or *msp*-2 gene. When alleles in each family were less than 20bp, they were considered the same.

Pearson Chi square test was conducted for statistical comparison of categorical variables. $P<0.05$ was used to test the level of statistical significance to accept or reject the hypothesis. Statistical tests were performed by using SPSS version 20.0 (SPSS Inc., Chicago, USA). In addition, geometric mean of parasite density was calculated by using the Microsoft excel 2016 version.

The expected heterozygosity (He) represents the probability of being infected by two different populations of *P. falciparum* with different alleles at a given locus was calculated by the formula;

$$He = \frac{n}{n-1}\left(1 - \sum p^2\right), \quad (2)$$

Where 'n' is, the number of the isolates analyzed and 'p' represents the frequency of each different allele at a locus.

4. RESULTS

4.1. Socio-demographic and Parasitological data of study participants

Of those 171 microscopically confirmed *P. falciparum* patients enrolled in the study; 139 (81%) and 148 (87%) samples were successfully amplified for *msp*-1 and *msp*-2 gene allelic variants, respectively. Parasite density ranged from 84 – 104,320 parasites/µl with a geometric mean of 5,654 parasites/µl. From the total male patients account for 70%. The age of the study participant ranged from 1 to 66 years with Mean ± SD of 27.0 ± 13.6* years. Of all the study participants, 57.4% were from urban inhabitants, and only 11% were having recent travel history to other malarious areas. Of all the study subjects, by occupation 76.9% *P. falciparum* malaria cases detected from students, daily laborers and farmers alone (Table 2).

Table 2: Socio-demographic characteristics and parasitological data of symptomatic malaria patients due to *P. falciparum* genotyped for *msp*-1 and *msp*-2 genes from Adama and its surroundings (September 2019 to August 2020)

Patient characteristics	**Sample genotyped**	**Chi square (X^2)**
Mean age (year)	27.0 ± 13.6*(SD)	
Age range (year)	1- 66	0.09
Sex ratio (Male/Female)	105/43	0.18
Residence (Urban/ Rural)	85/63	0.56
Travel history	17(11%)	0.076
Occupation		
Farmer	31(21%)	
Housewife	12(8%)	
Daily laborer	35(23.8%)	
Government employee	13(8.8%)	0.017
NGO employee	2(1.3%)	
Business man	7(4.7%)	
Student	47(31.7%)	
Geometric mean of parasitic density (P/µl) of blood	5,654	
Parasite density range (P/µl) of blood	84 – 104,320	
Parasitaemia level		
(50-499 P/µl of blood)	5 (3.4%)	
(500-4999 P/µl of blood)	63 (42.5%)	0.075
(5000-49,999 P/µl of blood)	75 (50.6%)	
(≥50,000 P/µl of blood)	5 (3.4%)	

4.2. Geometric mean of the parasite density across different age groups

Analysis of the geometric mean of *P. falciparum* parasite density across patients of different age groups has shown that school age children (5-14 years) carry disproportionate burden of the infection (Figure.7), showing decrement and relatively stable burden afterwards. However, the correlation between parasite density with patient's age is not statistically significant (Pearson's correlation = 0.12, P = 0.6).

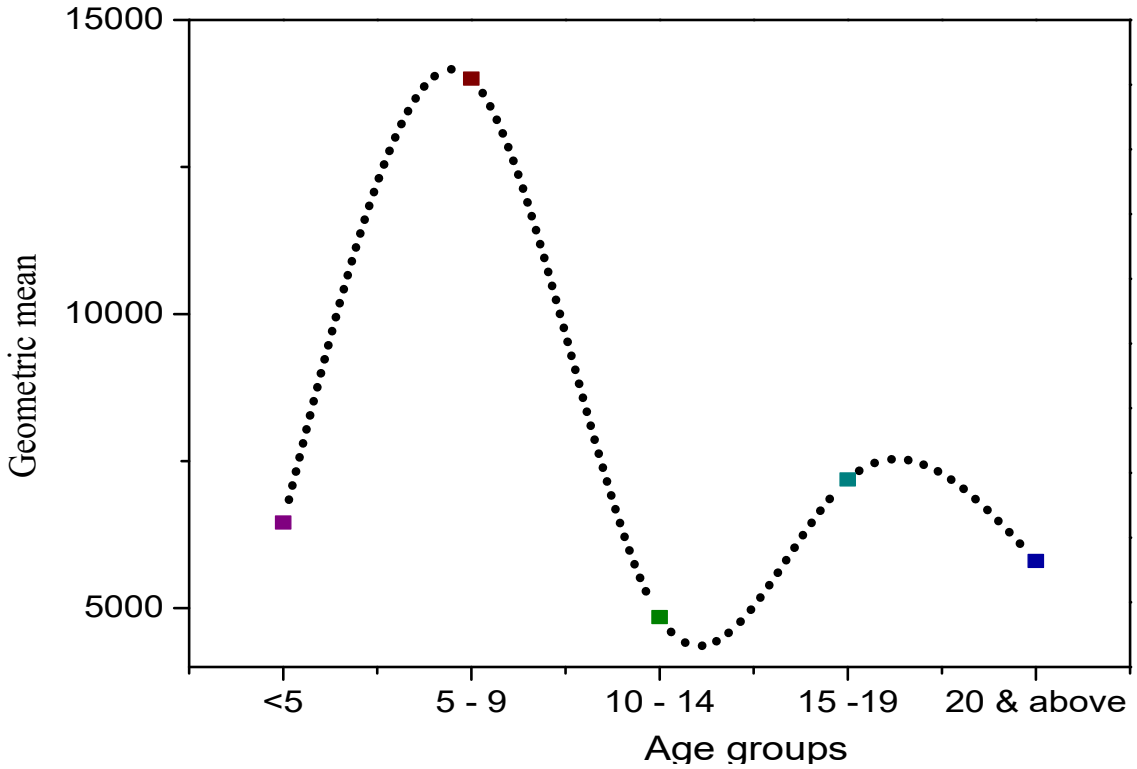


FIGURE 7: Relationship between geometric mean of parasite density with age groups of *P. falciparum* patients from Adama and its surroundings (September 2019 to August 2020).

Analysis of the geometric mean of *P. falciparum* parasite density showed significant variation [X^2 (12) = 22, P = 0.03] and [X^2 (18) = 36, P = 0.017] across different study sites (Figure.8) and occupation type (Table 2), respectively. However, no significant variation of parasitaemia level across seasons [X^2 (3) = 4.3, P =0.2], urban rural settings [X^2 (3) =4.8, P = 0.18], sex [X^2 (3) = 3.7, P= 0.29], age [X^2 (9) = 7.003, P= 0.63] and travel history [X^2 (3) = 1.7, P = 0.6]. This shows, human living conditions including occupational exposure and vector related factors like its abundance near human habitation highly contributed for such variation.

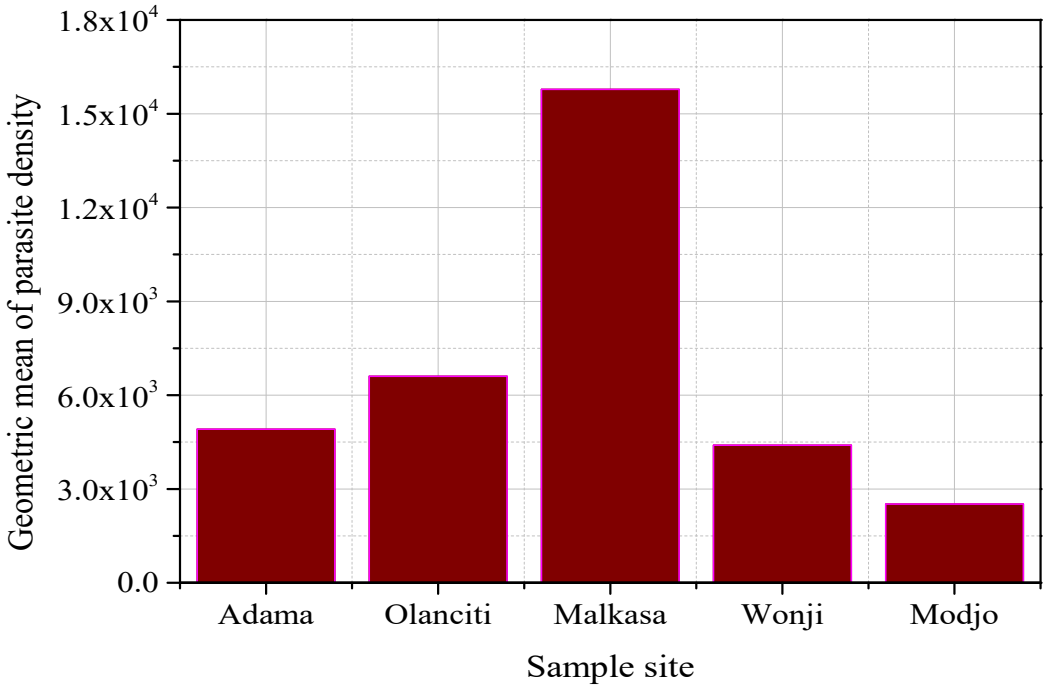


FIGURE 8: Geometric mean of the parasite density of *P. falciparum* patients across different sites in the study area (September 2019 to August 2020).

4.3. Allele frequency, genetic diversity and multiplicity of P. falciparum infections

Of 139 successfully genotyped *msp*-1 allelic families, MAD20 was detected with highest frequency (48.2%). Moreover, 19 (MAD20) and 1 (K-1) double infection were detected with their own variant forms. On the other hand, all (18) RO33 allelic family shown no double infection with their own variant form. Furthermore, of 148 successfully genotyped alleles of *msp-2* gene nearly revealed analogous transmission pattern, including 7 (3D7) and 2 (FC27) allelic forms having poly- infection type with their own variant forms. Polyclonal infections of *msp*-1 and *msp*-2 genes with different allelic families were observed in different proportion, as summarized in (Table 3).

Even though disproportionate burden of parasitaemia was observed in school age children, as depicted in (Table 4), the overall frequency of both *msp*-1 and *msp*-2 gene allelic variant among symptomatic patients increased with age groups. Similarly, MOI generally tends to increase with age.

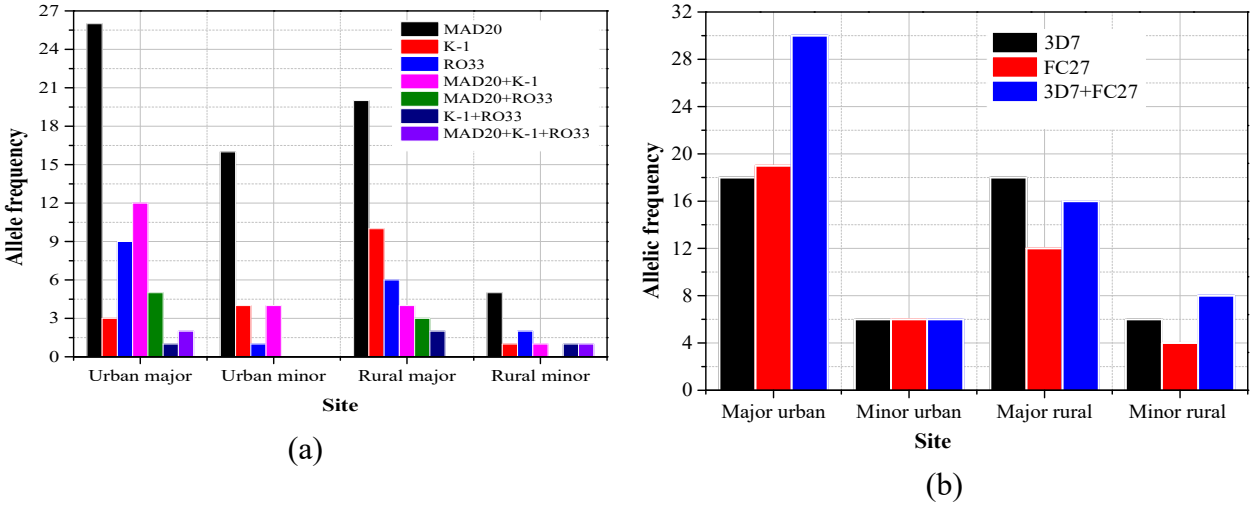
Table 3: Genetic diversity, allelic frequency and fragment size data of *P. falciparum* block 2 of msp-1 and block3 region of msp-2 genes from Adama and its surroundings (September 2019 to August 2020)

***msp-1* alleles (n = 139)**	**Allele frequency** n (%)	**Allele size (bp)**	**Number of alleles**	MOI	*He*
K-1	18 (12.9)	100 - 270	6		
MAD20	67 (48.2)	160 - 280	8		
RO33	18 (12.9)	100 - 200	5		
K-1+MAD20	21 (15.1)			1.42	0.65
K-1+RO33	4 (2.9)				
MAD20+RO33	8 (5.8)				
K-1+MAD20+RO33	3 (2.2)				
msp-2 alleles (n = 148)					
3D7	47 (31.8)	200 - 700	7		
FC27	41 (27.7)	250 - 700	10	1.46	0.68
3D7 +FC27	60 (40.5)				
Mean MOI		1.44 [range 1-5]			

Table 4: Distribution of *msp*-1 and *msp*-2 allelic variants, Average MOI and geometric mean of parasitic density among different age groups of malaria patients due to *P. falciparum* from Adama and its surroundings (September 2019 to August 2020)

S.N	Characteristics	Age groups (year)				
		<5	5 – 14	15 -24	>24	Total
1	MAD20	3	12	32	39	86
2	K-1	1	2	4	12	19
3	RO33	0	3	9	6	18
4	MAD20 + K-1	0	2	7	12	21
5	MAD20 + RO33	0	1	2	5	8
6	K-1 +RO33	0	0	0	4	4
7	MAD20 +K-1 +RO33	0	0	3	0	3
8	Total gel fragment	4	23	72	99	198
9	MOI	1	1.35	1.44	1.45	1.42
10	3D7	2	5	11	36	54
11	FC27	2	7	16	18	43
12	3D7 +FC27	1	6	28	25	60
13	Total fragment	6	24	87	100	217
14	MOI	1.2	1.4	1.6	1.4	1.46
15	Geometric mean of parasitic density	6455	7419	4324	6381	

Analysis of the urban-rural distribution of *msp*-1allelic variant during the major and minor malaria season in the study area shows high transmission patterns of MAD20 allelic variant (Figure. 9a). However, no statistically significant variation [X^2 (6) = 8, P = 0.2], in urban and rural areas during each season. Furthermore, similar pattern of overall MOI (1.43 to 1.45) was identified for the isolates from rural and urban areas, respectively. Similarly, analysis of the urban-rural distribution of *msp*-2 gene allelic variant during the major and minor malaria season in the study area (Figure.9b), depicts nearly analogous transmission patterns, showing no statistically significant variation [X^2 (2) = 1.1, P = 0.56], during each season. In addition, overall MOI revealed analogous pattern (1.44 and 1.48) for the isolates from rural and urban areas, respectively.



(a) (b)

FIGURE 9: Spatial and seasonal features of *msp*-1 (a) and *msp*-2 (b) gene allelic families from malaria patients due to *P. falciparum* from Adama and its surroundings (September 2019 to August 2020).

Sample isolates genotyped to analyze *msp*-1 allelic variants from the study sites shows the predominance of MAD20 allelic, which ranged from Adama (64.6%) to Modjo (16.6%). And the least prevalent allele was RO33, which varied from Modjo (11.1%) to Wonji (5.8%). Similarly, the spatial variation of the distribution of *msp-1* allelic variant across study sites was highly significant [X^2 (24) = 89, P ≤ 0.001], (Figure.10a). Likewise, the predominant *msp*-2 allele (3D7) ranged from (45.1%) in Adama to (19.2%) in Awash Malkasa. The spatial distribution of *P.*

falciparum msp-2 gene allelic variant across study sites indicated variation (Figure.10b), that was statistically highly significant [X^2 (8) = 29.6, P ≤ 0.001]. Thus, the distribution of *msp*-1 and *msp*-2 gene depicts heterogeneity in distribution of the parasite clones.

Comparison of number of allelic fragments for *msp* -1 and *msp* -2 genes by the number of positive sample for respective gene amplified shown highest MOI of *msp*-1 (1.8) and *msp*-2 (1.8) genes at Olanciti and Awash Malkasa and least (*msp*-1 = 1.35 and *msp*-2 = 1.3) genes at Wonji and Adama respectively (Figure. 11).

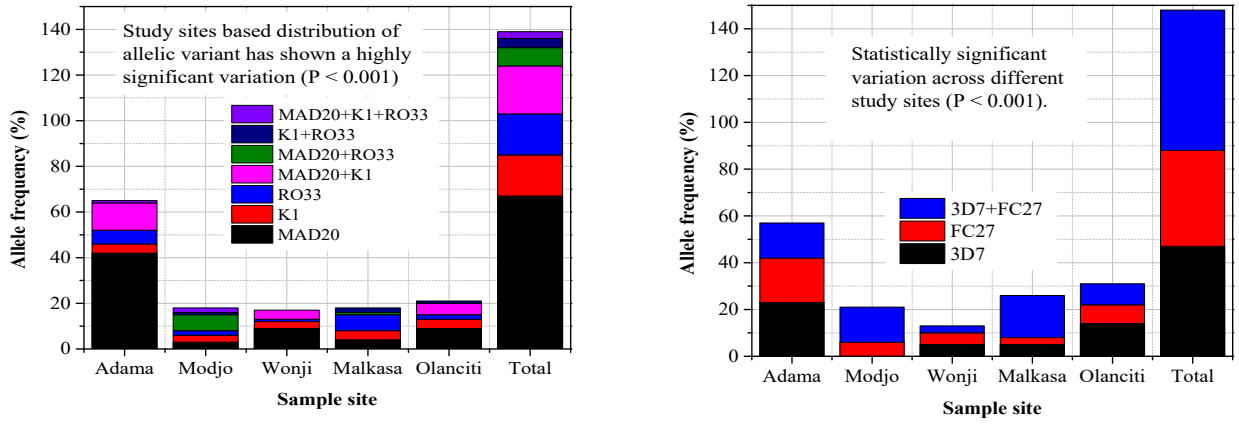


FIGURE 10: Comparison of *msp*-1 (a) *msp*-2 (b) gene allelic variants from malaria patients due to *P. falciparum* from Adama and its surroundings (September 2019 to August 2020).

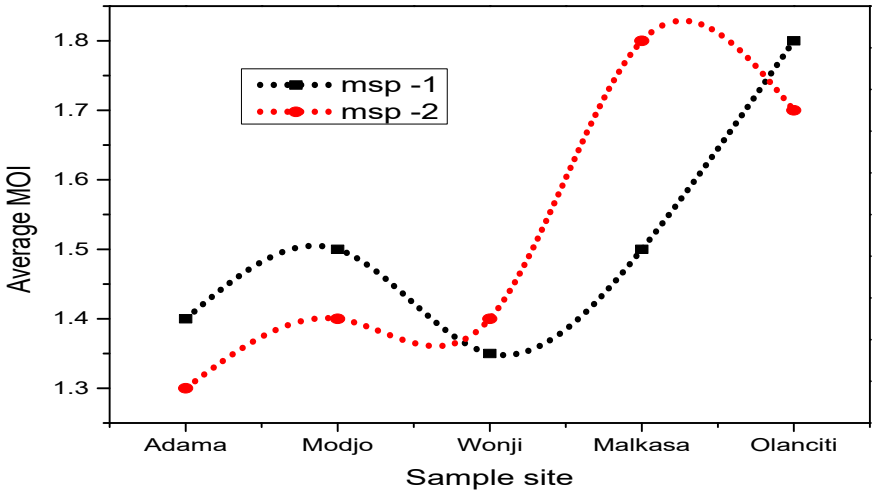


FIGURE 11: Trends of MOI with respect to *msp*-1 and *msp*-2 gene allelic families at different sites in the study area (September 2019 to August 2020).

5. DISCUSSION

Ethiopia has now moved forward in targeting nationwide malaria elimination program by 2030. For effective implementation of this strategic target, one of the key intervention strategies is improving malaria surveillance and response (FMOH, 2020). In this regard, molecular epidemiological studies like characterization of block 2 region of *msp-1* and block-3 region of *msp*-2 genes of the most prevalent and virulent malaria parasite *P. falciparum* provide comprehensive molecular evidence for effective disease surveillance that could ultimately be translated to core interventions in the control and elimination of malaria.

In the present study, socio-demographic data of *P. falciparum* clinical isolates from symptomatic patients revealed, consistence with our previous report (File and Dinka, 2020) and that of Golassa and White (2017), where the incidence of *P. falciparum* isolates in the study area was higher in male (71%). The major factors that may account for such higher malaria cases compared to female might be special risk of male individual to malaria infection from occupational and travel-related factors (FMOH, 2020), in Ethiopian tradition. Besides, the incidence of *P. falciparum* isolates in the present study was significantly related to occupation type ($X^2 = 0.017$) (Table 2) which is in agreement with the review of Degarege and his colleagues (Degarege *et al.*, 2019). This could be due to strong relation of malaria incidence with lower standard of living that contributed for the occurrence of 76.9% of all *P. falciparum* isolates in the present study from farmers, daily laborers, and students alone (Table 2).

Even though a number of factors may contribute to the fluctuation of parasitaemia level overtime in symptomatic patients, the geometric mean of microscopically detectable parasitaemia levels could be used to explain the finding of this study (Shekalaghe *et al*., 2005). Likewise, the present study revealed that; geometric mean of parasite density was disproportionately high in school age children (SAC), and shown decrement resulting relatively stable parasitaemia level afterward. The major factor that mainly contributed for higher parasitaemia level in SAC is delayed acquisition of protective immunity during this immunological transition age making this age group more vulnerable to malaria infection than adults *(*Makenga *et al.*, 2020). On the other hand, decrease in geometric mean of parasite density after SAC revealed in the present study (Figure. 7), indicates age dependent acquisition of natural immunity in malaria endemic setting. Moreover, in the present

study, there was no statistically significant correlation revealed between parasite density with age of the patients (Pearson's correlation = 0.12, P = 0.63).

Furthermore, contrary to the notion of age dependent immunity higher rate (85%) of *P. falciparum* cases in adolescent and adult age (>15 years) in this study could explain the importance of occupational and travel related factors for malaria transmission in favor of spatial heterogeneity model of reduced malaria transmission setting (Karl *et al.*, 2016). Study on geometric mean of parasite density (Figure. 8) per µl of blood sample of malaria patients from different sites, shown statistically significant variation (P = 0.03). Although the specific factors that contributed to such variation necessitate further study, host and vector related factors might have largely contributed to such spatial variation of parasitaemia level. Highest geometric mean of *P. falciparum* was detected from patient isolates from Awash Malkasa, where *msp*-1 allelic variant were predominantly (93%) mono-allelic form. The lack of intra species strain competition within the host (Sondo *et al*., 2019), might have contributed to relatively higher parasitaemia and eventually to disease severity. In the present study, microscopic detectable parasitaemia level from symptomatic patients greatly varied ≤500 (3.4%), 500- 49,999 (42.5%), 5000 – 49,999 (50.6%), and ≥50,000 (3.4%) per µl of blood (Table 2). Such variation in parasitaemia level could be due to sequestration, the variation in the level of protective immunity typical of hypo-endemic setting and blood cell polymorphisms (Shekalaghe *et al.*, 2005).

Characterization of *msp*-1 block-2 region revealed that of the total successfully genotyped *msp-1,* 26% of the isolates were having di-clonal and tri-clonal infections indicating lower transmission rate. Furthermore, 19 double infection of MAD20 and 1 double infection of K-1 alleles were detected, showing overall MOI of 1.42 and expected heterozygosity of 0.65 (Table 3). This finding differs from the report of northwestern Ethiopia and southwestern Ethiopia (Mohammed *et al*., 2018) and Abamecha *et al.*, (2020), where they reported 75% and 80% frequency of multi-clonal infections, MOI (1.8), *He* (0.79), 2.0 MOI and 0.43 *He,* respectively. This shows that malaria transmission in the present study area exhibits low genotype diversity within *msp*-1 locus compared with northwestern and southwestern Ethiopia. This could be due to the ongoing scale up of interventions, differences in local epidemiology, demographic and environmental conditions that might have resulted in such difference in genetic diversity patterns. Moreover, in the present study monoclonal and multiple clone infections gradually increased with age group (Table 4). This

finding is in congruent with the report from Burkina Faso (Soulama *et al.*, 2009), and Tanzania (Pinkevych *et al*., 2014), where they explained that episode of infection in children is common for very short duration and the duration of episode of infection increases with age contributing to mono-clonal and poly-clonal infection rate. Other reports suggested that multiple infections vary with parasite density, immunity status, the overall prevalence of infection in the population and transmission intensity as reviewed by Eldh *et al.* (2020).

In the present study, from 139 successfully amplified clinical isolates for *msp*-1 gene; 19 different length polymorphism of *msp*-1 allelic variants were revealed: 8 MAD20 (160 -330bp), 6 K-1 (100 -270) bp, and 5 RO33 (100 -220bp). The number of allele fragments identified in the present study might have been under estimated due to a number of limitations related to nested PCR amplification process and agarose gel electrophoresis (Abamecha *et al.*, 2020; Peakall and Smouse, 2012). Size polymorphism of *msp-1* allelic variant identified is slightly higher than the report from Chewaka district of southwestern Ethiopia (Abamecha *et al*., 2020) and Humera of northwestern Ethiopia (Mohammed *et al.*, 2018). But, less diverse than Kolla Shele district of southwestern part of Ethiopia (Mohammed *et al.*, 2015), and more or less similar to the report from Equatorial Guinea (Chen *et al.*, 2018, Bobo-Dioulasso of Burkina Faso (Somé *et al*., 2018). The major factor that may account for such variation could be; the scope of study sites covered and local malaria transmission patterns.

Gel-analysis of the present study revealed that: from 139 *msp*-1 amplicon 103 (74%) were mono-allelic infection, from which 20/103 (19.4%) were double infections of the same allelic form. The remaining 36 (26%) were poly-allelic type, with 15% for (MAD20 + K-1), 5.7% for (MAD20 +RO33), 2.8% for (K-1 +RO33), and 2.1% were for MAD20 +K-1 + RO33 type. The proportion of monoclonal infection was 48.2% MAD20, 13% K-1 and 13% RO33 (Table 3). This finding differ, from the report from southwestern Ethiopia (Abamecha *et al*., 2020; Mohammed *et al.,* 2015), where they reported that K-1 was the most prevalent allelic family. Similarly, report from Cameroon, Gambia, Nigeria and Gabon has shown that MAD20 allelic variant was least predominant (Metoh *et al.*, 2020; Zakeri *et al.,* 2005). On the other hand, in agreement with the report from northwestern part of Ethiopia (Mohammed *et al.,* 2018) and Sudan (Hamid *et al.,* 2016), of the three *msp-1* gene allelic families MAD20 was the predominant allelic type. The driving forces for such variation could be; evolutionary process like genetic drift, types and rate of

mutations, inbreeding, and the relative contribution of allelic variants in reproductive success, due to better adaptation to their host are some of the factors that might have contributed for such variation (Escalante, 2020; Sondo *et al*., 2020).

Urban rural and seasonal distribution of alleles (*msp* -1) showed high transmission patterns of MAD20 allelic variant, regardless of season and locality with no statistically significant variation (P = 0.2) (Fig.9a). Moreover, similar patterns of MOI (1.45, 1.43) were detected in urban and rural localities, respectively. This finding could be an evidence to show the similarity of malaria epidemiology in both urban and rural setting in the study area, demanding similar intervention endeavors. Furthermore, no significant correlation existed between *msp-1* gene multiple clone infections of *P. falciparum* with seasonal variation of malaria incidence (P = 0.8). In favor of this finding, report from southwestern Ethiopia (Getachew *et al.*, 2015), has shown that no correlation or negative correlation was found between the proportion of multi-clonal infections and parasite prevalence. On the other hand, report from high malaria endemic settings in Indonesia (Noviyanti *et al*., 2015) , and Papua New Guinea (Fola *et al*., 2017), shows the presence of positive correlation between the rate of polyclonal infections and annual parasite incidence. The possible reason for such contradictory report could be due to the variation in malaria burden. On the other hand, study sites based distribution of allelic variant has shown a highly significant variation (P < 0.001) (Figure. 10a and Figure. 10b). This could be due to the difference in geographical areas, intensity of local transmission pattern, differences in the age of the study population and variation in parasite density (Somé *et al.*, 2018; Yavo *et al*., 2016; Mwingira *et al.,* 2011; Färnert *et al.*, 2008).

Characterization of block-3 region of *msp-2* gene allelic variant of *P. falciparum* genetic profile revealed that, of the total successfully genotyped 47 (3D7) and 41 (FC27) *msp-2* gene, 59.4% (88/148) containing mono-allelic infection (Table 3), from which 10.2% (9/88) were containing ≥ 2 clones of the same allelic form. Report from maritime region of Togo (Awaga *et al*., 2012) and Ponte-Noire, Republic of Congo *(*Singana *et al.*, 2019) complement this findings. The number of *msp-2* genotypes detected for 3D7 (200 – 700 bp) and FC27 (250-700 bp) was 7 and 10, respectively. Although inherent limitation of the technique used may underestimate size polymorphic forms. The fragment size described in the current study is nearly comparable with the previous report from Republic of Congo (Singana *et al.*, 2019), Nigeria (Funwei *et al.*, 2018), Sudan (Hamid *et al.*, 2016), and northeastern Ethiopia (Mohammed *et al.*, 2021). Other reports

from Congo Brazzaville (Mwingira *et al.*, 2011), and northwestern Ethiopia (Mohammed *et al*., 2017) shown the predominance of 3D7 allelic family. Such inconsistency in *P. falciparum* allelic size polymorphism could be due to local malaria epidemiology and scope of sample population covered in the study. The rate of *msp*-2 multiple allele infection was 40.5% (Table 4), which partly reflects the transmission status of the parasite population in the study area, with the overall MOI of 1.46. This finding is lower than the previous report from southwestern Ethiopia (Abamecha *et al*., 2020), northwestern Ethiopia (Mohammed *et al*., 2017), Sudan (Hamid *et al.*, 2016), Cameroon (Metoh *et al.*, 2020), and Nigeria (Oyedeji *et al*., 2020); but somewhat higher than the previous report from northeastern Ethiopia (Mohammed *et al*., 2021), and Ghana (Adjah *et al.*, 2018). The variation in multi-clonal infection and multiplicity of infection could be due to the overall prevalence of infection in the population and the age of the individual (Soulama *et al.*, 2009). The overall MOI identified in the present study could serve as proxy of transmission intensity for targeted intervention in the region. Furthermore, the present study shown expected heterozygosity of (*He)* = 0.65, indicating low genotype diversity within *msp-2* locus in the study area, compared to the previous report from southwestern Ethiopia (Mohammed *et al.*, 2015), northwestern Ethiopia (Mohammed *et al*., 2017) and Malaysia (Atroosh *et al.*, 2011).

In the current study, analysis of *msp*-2 gene allelic variant in the urban and its surrounding rural area during the major and minor malaria transmission seasons (Figure. 9b), revealed analogous transmission pattern, having no statistically significant variation (P = 0.54). In complement with this finding Funwei and his colleagues (2018) reported similar level of genetic diversity in urban and rural areas of southwestern Nigeria. On the other hand, Soulama and his collegues (2018) reported that *msp*-2 allelic variant and its multiple infection vary from urban-rural location, transmission intensity and independent of seasonal change. The major factor that might have contributed to such inconsistent report could be differences in local epidemiology of malaria parasite flow between urban and its surrounding rural areas. In the present study, analysis of the variation of *msp*-2 allelic frequency across different age groups has shown increment in parallel with *P. falciparum* clinical prevalence during the study period (Table 4). Moreover, in this study MOI tends to increase with age when compared with younger children. This finding differ from the previous report from hyper-endemic area of Burkina Faso (Sondo *et al*., 2020), where they described the existence of negative relationship between multi-clonarity with patient age. However, consistent to the present finding, Pinkevych and his colleagues (2014) reported that, as

immunity develops, multiple infection rate seems to increase. Similarly, other report from Burkina Faso (Soulama *et al*., 2009), and Tanzania (Pinkevych *et al*., 2014), also explained that episode of infection in children is commonly for very short duration and the duration of episodes of infection increases with age contributing to the rise in multi-clonal infection rate in other age groups. Thus, the relationship between the levels of immunity with multi-clonarity needs further investigation. Moreover, complement with the report from Equatorial Guinea (Chen, 2018) the present study revealed the existence of direct relation with *msp*-2 allele frequency and MOI with the clinical prevalence of the *P. falciparum* cases (Table 4). This could be due to the level of acquired immunity in malaria endemic setting of the study area. Besides, district wise distribution of *msp*-2 allelic variant and multiple infections in the study area (Figure.10b) showed a highly significant variation ($P \leq 0.001$). This could be due to spatial heterogeneity of the local micro-ecological factors of the study sites under consideration (Smith *et al.*, 2012; Smith *et al*., 2010).

The characteristic feature of nearly moderate transmission setting in the study area revealed in the present study, has shown highly significant variation of allelic variant across different study sites. Such spatial heterogeneity in the variation of *P. falciparum msp*-1 and *msp*-2 gene allelic variant in the study area, consequently resulted in relatively higher MOI (1.8) and (1.7) at Olanciti for both *msp*-1 and *msp*-2 gene respectively and least at Wonji, with MOI (1.35) for both *msp*-1 and (1.4) for *msp*-2 genes (Figure. 11). Although specific factors that contributed to such variation needs further investigation, it could be due to vector related factors productivity of vector breeding sites and its distance from human dwelling (Karl *et al*., 2016; Bousema *et al*., 2010). Furthermore, various human related factors like occupational exposure, treatment-seeking behavior specifically when; people are living at a considerable distance from health facilities less frequently seek treatment (Karl *et al*., 2016; Bruce *et al*., 2000). Occupational exposure, level of acquired immunity, age of the patients, the status of bed net usage, time spent outdoors, quality of housing, beer consumption (Karl *et al*., 2016, Dabire and Costantini, 2010), parasite density (Sondo *et al*., 2019) and the like are some of the factors that might have contributed for such variation.

Even though, this is the first study to analyze *P. falciparum* genetic diversity in central Ethiopia, by using nested PCR amplification and gel-electrophoresis of its most polymorphic region of *msp*-1 and *msp*-2 genes. The study has some technical limitations. Firstly, the association between the observed dominant allelic families and disease severity of complicated malaria cases was not

examined, because all samples were collected from uncomplicated malaria patients. Secondly, the number of alleles might have been underestimated due to the inherent limitations of techniques used. Therefore, further characterization of such polymorphic region need to be designed by increasing the sample size, implementation of advanced techniques like microsatellite and/or DNA sequencing on samples isolated from symptomatic and asymptomatic cases for further study. However, I tried to manage such limitations following the established Standard Operating Procedures (SOPs) during the molecular work to generate comprehensive data that depicts the real picture of P. *falciparum* genetic diversity from symptomatic patients in the study area.

6. CONCLUSIONS AND RECOMMENDATIONS

The present study revealed moderate genetic diversity of *P. falciparum* clinical isolates found in the study area, when compared with similar study conducted in southwestern, northwestern and northeastern parts of Ethiopia. Moreover, high frequency of MAD20 allelic variant of *msp*-1 gene was detected, and nearly analogous transmission patterns of *msp*-2 allelic variant were detected during the study. Furthermore, the present study revealed the absence of significant variation in allele frequency in both urban and its rural counterpart and similar allele frequency during the major and minor malaria seasons, reflecting frequent inbreeding among the existing parasite strains in both settings.

Therefore, to sustain the national and regional declining phase of malaria transmission in Ethiopia, in addition to the classical epidemiological study approach, the ongoing malaria control and elimination strategy should be accompanied with similar molecular surveillance for targeted intervention. The findings indicated in the study reveals strong evidence for the presence of similar malaria epidemiology in urban and rural location of the study area demanding similar intervention endeavors. The driving force for such selective advantage for MAD20 allelic variant and its association with disease severity level in our study area demand further investigation. Thus, the findings described in the present study will serve as a baseline molecular evidence for further research on areas having similar malaria epidemiology to make the control and elimination efforts effective.

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8. APPENDICES

Appendix 8. 1 Standard Operating Procedure for DNA Extraction (template preparation) from Dry Blood Spot (DBS), by using Tween® 20 /Chelex method.

Adama Science and Technology University

School of Applied Natural Science

Department of Applied Biology.

Laboratory set-up: Malaria Research Laboratory at Aklilu Lemma Institute of Pathobiology, AAU

Supervisors:

Professor Hunduma Dinka

Dr. Lemu Golassa

By Temesgen File (PhD Student)

January, 2020

Adama

Ethiopia

Title: DNA Extraction by using Tween® 20 / Chelex method:

Objective: To extract DNA of *P. falciparum* from the blood spotted on Whatman filter/DBS kit.

Introduction:

Extraction of DNA from DBS previously collected on Whatman filter paper/DBS kit, dried for a minimum of 4 hours and stored in the freezer at -20°C, can be conducted by using 0.5% Tween® 20 /Chelex method. Tween® 20 is used to lyses the erythrocytes, due to its emulsifying property. Whereas Chelex, is used to bind and remove metal ions like Magnesium (Mg^{+2}), to stabilize DNA in the downstream procedures. The protocol is optimized to extract DNA from 3-5mm diameter punch of DBS to obtain sufficient quantity of DNA for Nested amplification. This protocol is fast, efficient in detecting low parasite density, cost effective, and the procedure is relatively less complicated. However, it is prone to contamination, unless otherwise the standard protocol is strictly followed.

Materials required:

- Puncher/Scissors
- Forceps
- 0.5ml micro-centrifuge tubes/ Eppendorf tube/
- 1.5ml micro-centrifuge tubes
- Micro-centrifuge
- Racks
- Micropipettes (different size)
- Micropipette tips (Different size)
- Shaker
- Bunsen burner
- Analytical balance
- Water bath (at 96ºc)
- Refrigerator
- Permanent marker
- M-tork (tissue paper)
- Gloves

Reagents

- 90-100% ethanol
- 0.5% Tween 20 in PBS prepared fresh or stored at 4°C or -20°C.
- 6% Chelex-100 in DNA'ase/RNA'ase free water stored at room temperature.
- Distilled water DNA/RNA'ase free.
- PBS solution in DNA/RNA'ase free water, stored at 4°C.

Preliminary work for the procedure:

- Clean and disinfect the designated area/work bench.
- Collect and organize all the necessary materials.
- Make sure that the working reagents are prepared.
- Cover the cleaned and dried workbench with M-tork (tissue paper).
- Select a reasonable quantity of DBS samples from the -20 freezer to run the extraction.
- Arrange DBS sample, disinfectants, and /Bunsen burner in appropriate place.
- By using waterproof ink, Label the extraction tubes, and Eppendorf tubes clearly and consistently with the same ID with DBS sample.
- Enter sample information on Logbook.

Procedure:

Day One:

1. Disinfect the puncher/scissors, before and after the punching by placing in 70% alcohol and passing through the Bunsen burner flame.
2. By using the puncher/scissor cut 2-3 pieces or 3-5mm punch of a single blood disc from blood covered areas of a blood spot. Add the discs to the 1.5ml extraction tubes with forceps.
3. Sterilize the puncher and forceps by dipping briefly in ethanol, and flaming using the Bunsen burner. Ensure no alcohol remains on the tool, but also that the tool is not too hot when cutting occurs. By ensuring prompt flaming, overheating can be avoided.
4. Add 1ml of PBS (prepared from 8gm of NaCl, 0.2gm of KCl, 1.15gm of $NaHPO_4$, 0.24gm of K_2HPO_4) containing 0.5% Tween® 20 to the corresponding 1.5 ml microfuge tube.
5. Make sure that all the tubes are well tightly closed.

6. Mix well and make sure that the filter paper is completely immersed inside the buffer (PBS).
7. Put the tubes (in a rack) on a shaker, use an intermediate shaking speed & make sure that all the discs are freely moving in the tubes & the rack is properly stabilized.
8. Incubate the microfuge tube or the 96 well micro titer plate at 4°C overnight, making sure that filter paper elution is successful (by observing the color of the filter paper).

Day Two:

9. Vortex and centrifuge the tubes at high speed (13,000rpm) for 10 minutes. Confirm that your filter paper discs are white. If not, this protocol might not yield the best quality of DNA.
10. Discard the liquid content 0.5%Tween 20, by using pipettor, while aspirating 0.5%Tween 20, take care not to touch the filter paper.
11. Vortex and add 1ml of cooled PBS to each tube from which saponin is removed and leave it at 4°C for 30 minutes.
12. Centrifuge at 13,000rpm for 5 minutes. Discs MUST now be at the bottoms. And remove as much liquid as possible by using pipette tips (P1000).
13. Add 300 µl of 6% chelex in TE buffer.
14. Vortex and incubate in the water bath at 95ºC for 12 minutes. Open the cap to release pressure after two minutes and vortex every three minutes.
15. Centrifuge at 13,000rpm for 8minutes
16. Transfer the supernatant (DNA template) in to clean labeled 1.5ml centrifuge tubes.
17. Centrifuge again at 13,000rpm for 1 minute
18. Transfer the liquid to clean labeled 0.5 ml centrifuge tubes. Avoid chelex particles reside at the bottom. Note that good quality DNA should be clear color, and may be light yellowish some times.
19. Label the microfuge tubes in the box consistently and the store at -20°C.
20. Measure the quality of each extracted DNA by using Nano Drop.

Remark 1: PBS Preparation

- PBS tablet ---------- One tablet

- Distilled water ------ 200ml
- Procedure for PBS preparation:

i. Measure 200ml distilled water in measuring cylinder.

ii. Transfer 200ml distilled water in the flask and add one tablet PBS

iii. Dissolve the tablet by using electronic stirrer.

iv. Autoclave dissolved PBS solution.

Remark 2: Preparation of 0.5% Tween/PBS solution.

i. For (175 samples), we need ------ 0.85ml Tween 20

ii. PBS solution ---------- 100ml

Remark 3: Preparation of 6% Chelex

i. Chelex 100 resin …… 6gm

ii. DNase/RNase free water ------- 100ml

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

Ex. To calculate the amount of chelex to be used for 6 samples, including the control one.

- In the procedure, since we need to add 300µl of 30% chelex, for 6 sample, 6X 300µl = 1800 µl (1.8ml = 2ml).
- For 30gm chelex , we need 100ml DNase/RNase free water, for 2ml total volume, we need to dissolve $\frac{2ml X 30gm \text{ Chelex}}{100ml}$ = 0.6gm Chelex

Similarly, in this protocol we need 30 µl of 30% chelex, then for 140 samples. The amount of chelex needed in gm will be;

140x300 µl= 42,000 µl, i.e. 42ml.

For 30gm chelex, we need, $\frac{42ml\ X\ 30gm}{100ml}$ = 12.6gm

Appendix 8.2 Standard Operating Procedure for PCR based Plasmodium species identification.

Laboratory set-up: Malaria Research Laboratory at Aklilu Lemma Institute of Pathobiology, AAU

Adama Science and Technology University

School of Applied Natural Science

Department of Applied Biology.

Supervisors:

Professor Hunduma Dinka

Dr. Lemu Golassa

By Temesgen File (PhD Student)

January, 2020

Adama

Ethiopia

Title: PCR based species identification of *P. falciparum*

Objective: To evaluate Geimsa stained microscopic detection level of *P. falciparum* by using molecular method.

Introduction:

So far, microscopic examination is a primary method for malaria parasite detection and species identification. Even so, skillful and experienced microscopist may sometimes miss the parasite or fail to correctly identify the species in molecular epidemiological studies. The recent advance on PCR based molecular detection targeting the 18s rRNA gene increases the sensitivity and specificity of malaria parasite detection when compared with microscopy.

Amplification procedure (refer SOP3 for the detail)

Amplicon should be kept at 4^0C, until running 2% agarose gel electrophoresis for visualization of the appropriate band (refer SOP4) for the details.

Remark: The expected amplicon size of *Plasmodium falciparum* in 205 bp

Appendix 8. 3 Standard Operating Procedure for Nested PCR amplification of *msp-1* & *msp-2* of P. falciparum from Dry Blood Spot (DBS).

Adama Science and Technology University

School of Applied Natural Science

Department of Applied Biology

Laboratory set-up: Malaria research Laboratory at Aklilu Lemma Institute of Pathobiology, AAU

Supervisors:

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January, 2020

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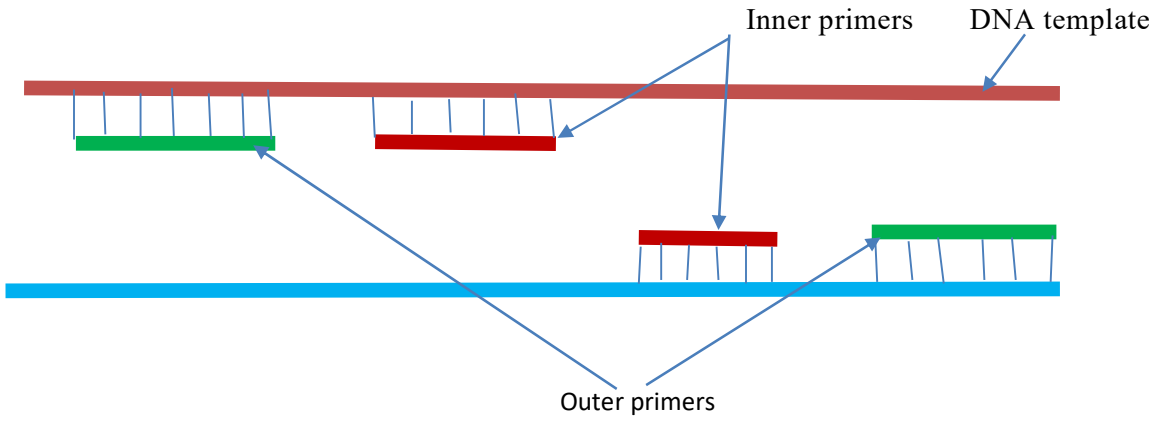
Ethiopia

Title: Nested PCR amplification of *msp-1 &msp-2* of *P. falciparum* from Dry Blood Spot (DBS).

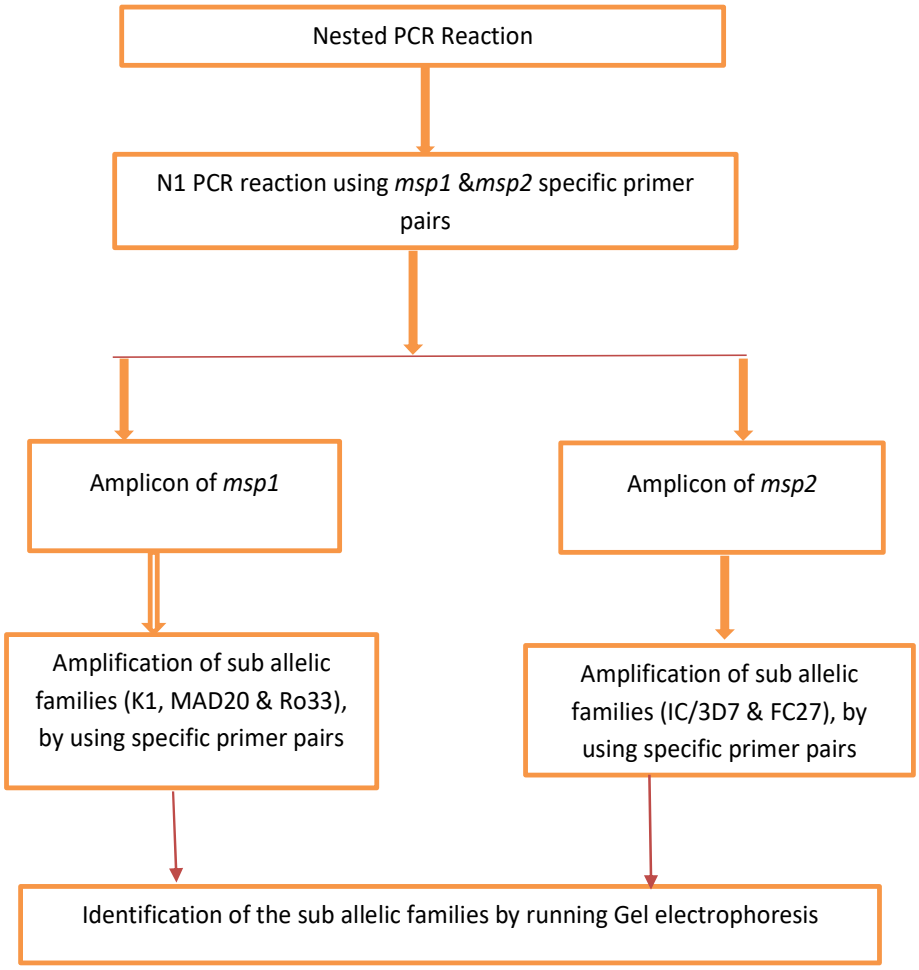
Objective: To amplify *msp-1 & msp-2* genes of *P. falciparum* and the allelic families of each by using Nested PCR protocol.

Principle: Nested PCR is a modification of conventional PCR to increase specific amplification of the target gene. Two set of (outer primers) are used to amplify the longer fragment of msp1 & msp2. The amplicon of the first set of the PCR product (Nest 1) is the site for binding for the inner set of primers during Nest 2 amplification, as summarized underneath.



Nested PCR amplification and analysis of *P. falciparum msp-1* and *msp-2* is informative for the identification of genetically distinct parasite sub population. In nest 2 amplification three pairs of primers are used to amplify K1, MAD20, & RO33 allelic families of *msp-1*, and two pairs of primers are used to amplify IC/3D7 & FC27 for *msp-2.* The schematic procedure of nested PCR amplification is as summarized below;

Schematic summary of nested PCR protocol



Materials required:

- Thermocycler
- Micro pipettes (with different size)
- Micropipette tips (with different size)
- PCR tube/plate
- Eppendorf tube (1-2 ml for master mix preparation)
- Mini centrifuge
- Plastic sealer for PCR plate
- Waterproof indelible ink

PCR reagent

- Molecular Biology grade water (DNase, RNase, & Protease not detected (Fisher Bio Reagent®).
- 5 x FIREPol® Master Mix to amplify msp1 &msp2 genes and their allelic families. The FIREPol® DNA polymerase containing 1mM dNTP each, i.e. 1x PCR solution contain 200µM dATP, 200µM dCTP, 200µM, dGTP and 200µM dTTP. 1x PCR solution containing 2.5 mM $MgCl_2$, 0.4 M Tris HCl, 0.1M $(NH_4)_2$ SO_4, 0.1% w/v Tween 20.
- 50bp DNA ladder (Invitrogen, by thermal Fisher-scientific), was used to analyze band size of the allelic families.

Appendix 8.4. PCR formulations

PCR formulation for Plasmodium species-specific amplification (Optimization)

PCR ingredient	Stock concentration	Volume per reaction (µl)	Total volume (µl)	Remark
Nuclease free water	-	13	13x10= 130	
Primer forward	0.25µM	0.5	5	
Primer reverse	0.25µM	0.5	5	
5x Hot five Pol.MM	10µM	4	40	
Aliquot MM		18		
DNA template		2		
Total volume		20		

Number of sample= 5 Pos C =2 Blank =1 Pipetting error= 2 Total= 10

PCR formulation for *P. falciparum msp*-1 & *msp*-2 gene amplification (N1) (Optimization)

PCR ingredient	Stock concentration	Volume per reaction (µl)	Total volume (µl)	Remark
Nuclease free water	-	11	11x10= 110	
Primer forward	0.25µM	0.5	5	
Primer reverse	0.25µM	0.5	5	
5x Hot five Pol.MM	10µM	4	40	
Aliquot MM		16		
DNA template		4		
Total volume		20		

Number of sample= 5 Pos C =2 Blank =1 Pipetting error= 2 Total= 10

PCR formulation for *P. falciparum msp*-1 gene (allelic family and *msp*-2 gene (allelic family) amplification (N2) (Optimization)

PCR ingredient	Stock concentration	Volume per reaction (µl)	Total volume (µl)	Remark
Nuclease free water	-	13	13x10= 130	
Primer forward	0.25µM	0.5	5	
Primer reverse	0.25µM	0.5	5	
5x Hot five Pol.MM	10µM	4	40	
Aliquot MM		18		
DNA template		2		
Total volume		20		

Number of sample= 5 Pos C =2 Blank =1 Pipetting error= 2 Total= 10

Procedure to run nested PCR amplification

A. Nest 1
    1. Add all PCR regents except the template DNA in the first Eppendorf tube
    2. Aliquot 16µl of the master mix in to PCR plate/tube
    3. Add 4µl of the DNA template to the aliquot
    4. Load samples in to thermal cycler, and program it as follows;

	PCR program (N1)			
Step/cycle	**Time**	**Temperature(°C)**	**Operation**	
1st step	3min	95	Initial denaturing	x1
2nd step	1min	94	Denaturing	**x30**
3rd step	1min	58	Annealing	
4th step	2 min	72	Extending	
5th step	5min	72	Extending	

B. Nest 2 amplification

1. Add all PCR regents except the template DNA in the first Eppendorf tube
2. Aliquot 18µl of the master mix in to PCR plate/tube
3. Add 2µl of the DNA template to the aliquot
4. Load samples in to thermal cycler, and program it as follows;

	PCR program (N2)			
Step/cycle	**Time**	**Temperature(°C)**	**Operation**	
1st step	3min	95	Initial denaturing	x1
2nd step	1min	94	Denaturing	**x30**
3rd step	1min	58	Annealing	
4th step	2min	72	Extending	
5th step	5min	72	Final Extending	

Visualization of the amplified DNA is by running 2% gel electrophoresis, stained with Ethidium Bromide.

Appendix 8. 5 Standard Operating Procedure for gel-electrophoresis to visualize Nested PCR amplification of *msp-1* & *msp-2* of *P. falciparum* from Dry Blood Spot (DBS).

Adama Science and Technology University

School of Applied Natural Science

Department of Applied Biology

Laboratory set-up: Malaria research Laboratory at Aklilu Lemma Institute of Pathobiology, AAU

Supervisors:

Professor Hunduma Dinka

Dr. Lemu Golassa

Temesgen File (PhD Student)

January, 2020

AIPB, AAU

Ethiopia

Title: Preparation and running **of** Agarose Gel-electrophoresis, to analyze *msp*-1 and *msp*-2 of *P. falciparum*

Objective: To visualize *msp-1* & *msp-2* genes and its allelic families isolated and amplified from *P. falciparum* by Nested PCR protocol.

Principle:

Gel electrophoresis is the standard molecular laboratory technique for separating DNA by size. An electrical field is used to move the negatively charged DNA through an agarose gel matrix toward a positive electrode. Shorter DNA fragments migrate through the gel more quickly than longer ones. Thus, the approximate length of a DNA fragment can be determined by running it on an agarose gel alongside a DNA ladder.

Materials:

- Casting tray
- Well combs
- Voltage source
- Gel box
- UV light source
- Microwave

Reagents:

- TAE buffer
- Agarose
- Ethidium Bromide (stock concentration of 10 mg/mL)

Procedure:

1. Add 2 g of agarose in 100 mL of TAE buffer, to make a 2% gel.
2. Mix very well, boil in the Microwave for 1-3 min, at an interval of 30-40second stop, and swirl it.

3. Let agarose solution cool down to about 50- 60°C (about when you can comfortably keep your hand on the flask).
4. Add 6 µl Ethidium Bromide when the gel cooled down, approximately between 50-60 ^{o}C, touchable by hand. EtBr binds to the DNA and allows you to visualize the DNA under ultraviolet (UV) light.

Note: Ethidium bromide a mutagen; take a maximum care and use gloves.

5. Pour the agarose into a gel tray with the well comb in place. Pour slowly to avoid bubbles that will disrupt the gel. Any bubbles can be pushed away from the well comb or towards the sides/edges of the gel with a pipette tip.
6. Place newly poured gel at 4 °C for 10-15 minutes OR let sit at room temperature for 20-30 minutes, until it has completely solidified.

Sample Loading

7. Carefully remove the combs from the gel
8. Put the gel on to the cast tray containing the gel into an electrophoresis tank and the tank should be filled with TAE buffer until the gel covered.
9. A drop 6X loading dye to each well.
10. Take 5 µl DNA ladder, and loaded on the first well (a molecular weight ladder into the first lane of the gel).
11. Take 5µl sample and load on the next consecutive wells.
12. Load at least one positive control & one negative control in the last lane
13. The gel tank should be covered with its cover and connect the power cables to the power supply; making sure that the cables will be connected to the right hole on the power supply. And also note the gel is put on the right direction that the insertion lines or wells towards the anode (red cable)
14. Run the gel at 120V & 400 ampere for 45-60 min, i.e. until the dye line is approximately 75-80% of the way down the gel.
15. The gel should be carefully taken out and the picture should be taken using the gel documentation apparatus.

Photographing of the agarose gel with UV light

1. The gel will be taken out from the gel cast carefully with the spoon.
2. The UV box will be cleaned with alcohol.
3. Carefully put the gel in to the UV box and the UV box should be closed.
4. Attach the UV box to the computer to take the photo, the picture should be saved.

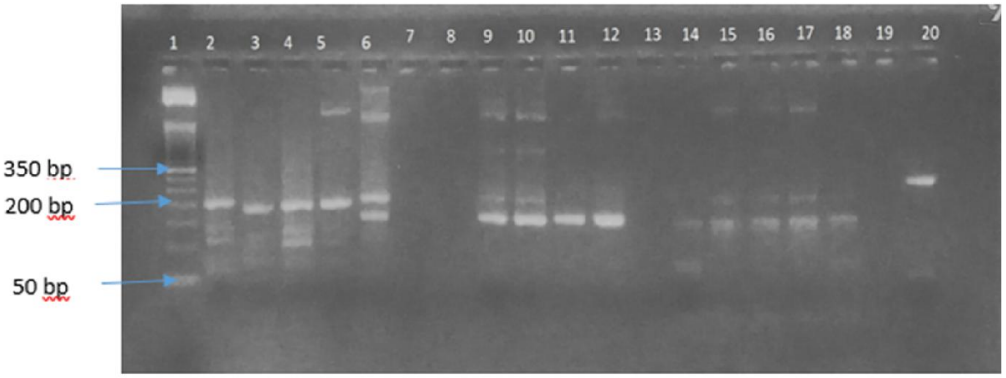
Reading the gel

The presence, absence and intensity of the bands will be checked. Comparison will be made with the reference sample so as determine the amount of DNA produced.

Interpretation of gel lane

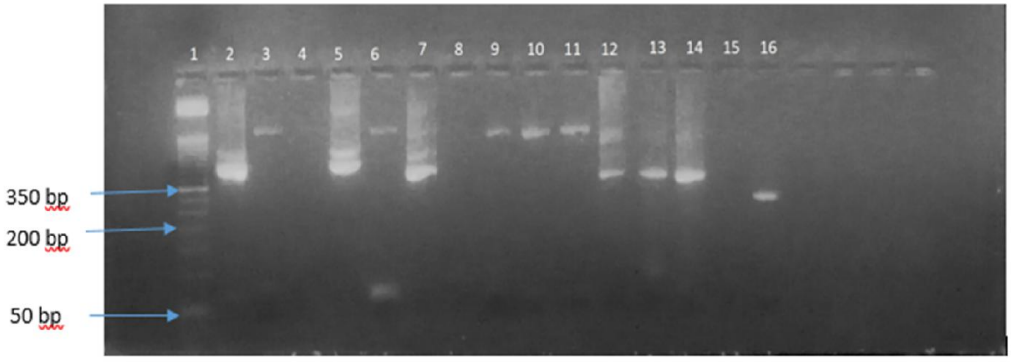
- The expected amplicon product of Genus Plasmodium is 1200bp.
- The expected amplicon product of *P. falciparum* is 205bp.

Appendix 8.6 (A) and 8.6 (B): Sample of gel electrophoresis of (a) *msp-1* and (b) *msp-2* allelic variants of *P. falciparum.*



A

Lane 1: 50 bp ladder, Lane 7 Negative control for MAD20, Lane 13 negative control for K-1, Lane 19 negative control for RO33, Lane 20 is allele specific positive control (3D7). Lane 2,3,4,5,6 are positive for MAD20, Lane 9,10,11,12 are positive for K-1, and Lane 15,16,17,18 are positive for RO33 allele.



B

Lane 1: 50 bp ladder, Lane 8: Negative control for FC27, Lane 15: Negative control for 3D7 allele, Lane 16 allele specific positive control (3D7), Lane 2, 3,5,6,7 are positive for FC27, and Lane 9,10,11,12,13,14 are positive for 3D7 allele.

Appendix 8. 6 Patient Information Sheet

Dear Participant,

Background and aim of the study

This is a PhD dissertation project entitled; the "**Genetic diversity and Multiplicity of infection of *Plasmodium falciparum* clinical isolates from Adama and its surroundings, Oromia, Ethiopia",** Adama Science and Technology University (ASTU), School of Applied Natural Sciences, Department of Applied Biology conduct the study. The interest to study malaria in Adama and its surroundings is because malaria is still a major public health problem in Ethiopia. Even the problem is over looked in urban setting with the presumption that urbanization greatly reduces the disease burden, although this is not always true in sub Saharan Africa (SSA) to which Ethiopia belong. The major aim of the study is to study genetic diversity, multiplicity of infection of *P. falciparum* in the study area.

Study Participants

Any uncomplicated clinically confirmed malaria patient due to *P. falciparum* persons working or living within the catchment area of Adama city administration and its nearby districts who attend blood test for malaria diagnosis in selected health facilities, can participate in the study. It is not necessary to participate in the study, the study participants are free to make a voluntary decision to participation or not to participate. There is no penalty for refusing to participate in this study. In case the study participant agrees to participate and later change their mind about it, they are free to withdraw from the study. In this study, following the identification of *P. falciparum* in the blood by microscopy, blood sample were collected by the researcher or experienced laboratory technologies for Dry Blood Spot (DBS) preparation by finger pick method for further molecular study in advanced molecular Biology laboratory.

Risk associated with Blood Collection

Finger prick blood sample collection will be performed by well-experienced professionals by using sterile equipment. The procedure does not cause any harm to you, except some feeling of discomfort while your blood is being collected. In case of any injury of yourself (your child), we will provide medication at our cost in the local health facility.

Benefits

By taking part in this study or not, you will not get a direct benefit but your participation will contribute to the generation of new knowledge on malaria in Ethiopia, and reduce malaria related morbidity and mortality. Stakeholders like Federal Ministry of Health (FMOH), Oromia Health Bureau, Adama City Administration, district health offices included in the study and the community will be benefited from the study. The finding of the study will also support malaria control and elimination efforts in Ethiopia.

Confidentiality

Any information that we collect about you or your child during the study will be kept confidential. Information about your identity will be coded and kept in a secured place and deleted after data processing. It is only the principal researcher (PR) who have a chance to relate the epidemiological data with malaria related clinical information. Your answers to interview will be registered in questionnaire format and used only for this research. The questionnaire will be answered by the child's guardian/parent if he/she is less than 12 years of age.

Sharing the Result

At the end of this research work, we will write the research report and communicate the major finding to the academia through publications. Whatsoever, the reports will never disclose your name or identity. We assure you the confidentiality of such information.

Contact Address

1. **Temesgen File: Address: Adama Science and Technology University, School of Applied Natural Sciences, Department of Applied Biology, Email: temesgenfile@gmail.com**
2. **Prof. Hunduma Dinka: Adama Science and Technology University, School of Applied Natural Sciences, Department of Applied Biology, Email:** dinkahu@gmail.com
3. **Dr. Lemu Golassa: Addis Ababa University, Aklilu Lemma Institute of Pathobiology, Addis Ababa, Telephone (mobile): +251-911-371981, E-mail:** lgolassa@gmail.com; lemu.golassa@ju.edu.et

Thank you for your participation and cooperation in this study,.

Appendix 8. 7 Study participants/guardian consent /assent form

Information related to malaria patient, due to *P. falciparum*

Name of the malaria patient (participant) _____

Age_____, Sex _____, Code Number_____

Study site /Health facility name _____

I confirm that I have got sufficient information on the purpose of the research project the "**Genetic diversity and Multiplicity of infection of *Plasmodium falciparum* clinical isolates from Adama and its surroundings, Oromia, Ethiopia**". The major target is to contribute the control and eliminate effort of malaria in Ethiopia. I agreed to give necessary information and blood sample needed for the research. The researcher or laboratory technician informed me that finger prick to collect the blood sample would not cause any harm to me. In addition, I understood that the data will be used only for research purpose, and the personal information of mine will be kept strictly confidential. All information are explained to me in a language that I most understand, and the information are all clear and I finally agreed to participate in the research and contribute to the control and elimination effort of malaria in Ethiopia. Finally, I decided to give all the necessary information and blood sample that is to be collected by finger prick, and signed this consent/assent form.

Are you a patient or Guardian? (Please put √ mark, to specify your position)

Patient_____ Guardian _____

Nana of the participant _____ Signature _____ Date _____

Name of an investigator obtaining consent/assent form _____

Signature _____ Date _____

Name of witness _____ Signature _____ Date _____

Appendix 8. 8 Research data and Consent form

Microscopic slide/DBS Serial number _____

Note: The following questionnaire is set only for *P. falciparum* malaria patients. If the patient is child, their guardian may respond to the questionnaire by using

	Epidemiological data & Lab result	Response (Please give √) mark, where appropriate
1.	Age of the patient is_____ years old.	
2.	Sex	
	M	
	F	
	Residence	
	Urban (Pl. specify kebele name)	
	Rural (Pl. specify kebele name)	
	Others place (Pl. specify)	
3.	Occupation	
	Farmer	
	House wife	
	Daily laborer	
	Government employee	
	NGO employee	
	Trader	
	student	
4.	Patients travel history in the last 30 days to malarious area	
	Yes (specify the place)	
	No	

*To be filled by lab technologist

Patient Blood film result

Positive, Pf_____.

Quantity of asexual stage per 200/WBC_____

Laboratory Technologists **Patient/Guardian**

Name _____ Name_____

Signature _____ Signature_____

Date _____ Date _____

Appendix 8. 8 Instruction for DBS preparation by Data Collector (DC)

1. Check the contents of DBS specimen collection kit.
2. Make sure the placement of the necessary materials like (Alcohol Wipe, Blood Specimen Collection Card, Gauze Pads, Bandage, and Safety Lancet) are on a dry surface.
3. Write the date and time in the space provided on the Blood Specimen Collection Card.
4. Open the collection card and fold the cover flap back, away from the collection circles. Lay the card down flat so that five circles are facing up.
5. Open the Alcohol Wipe and Gauze Pads.
6. Wash your hands with soap and warm water. Dry your hands with a clean towel.
7. Select the finger you are going to prick. Choose the middle or ring finger from your non-dominant (weak) hand.
8. Choose a puncture site off the center of the fingertip. In choosing a puncture site, avoid callused (hardened) areas of the finger.
9. Twist the cap off the Safety Lancet and throw the cap away. A tiny blade is hidden inside the lancet and is activated when you press on it. Because the lancet can only be used once, we have sent a spare one in this kit.
10. Wipe the tip of the finger you have chosen to prick with the Alcohol Wipe.
11. Stimulate blood flow to the finger by vigorously shaking your hand back and forth below the waist for a few seconds while standing.
12. Place the hand with the finger you have chosen to prick on a flat surface with your palm facing up.
13. Place the tip of the Safety Lancet against your fingertip. Press the lancet down firmly against your finger until you hear a click.
14. Wipe away the first drop of blood using the Gauze Pad.
15. Do not squeeze your fingertip hard. Apply pumping pressure at the base of the finger and then massage towards the tip, as in a milking action.
16. Turn your hand over and allow drops of blood to collect at the puncture site. Position the finger you pricked over the first circle on the collection card.
17. Once a large drop of blood has formed, touch the blood drop gently inside the first circle on the collection card. Try not to touch the collection card with your finger.

18. Allow the blood to completely fill the first circle before moving on to the next circle. It is very important you FILL up the circle COMPLETELY before you move to the next. If a circle is not completely filed with one drop, allow additional drops to fall onto the same circle. You may put extra blood outside the circle but the circle itself needs to be completely filed.
19. Fill the remaining circles one at a time. Once you have left a circle, do not go back. Fill at least 4 circles.
20. Once you have collected your specimen, use the second, clean Gauze Pad to wipe the remaining blood off.
21. IMPORTANT! Allow the card with your blood specimen to fully air dry on a flat surface such as your kitchen table or counter for at LEAST 4 hours, but no more than 12 hours at room temperature. Do not close the flap. Keep the card away from direct sunlight or any heat source or moisture. Keep the card away from children and pets. Do not touch or smear the blood spots.
22. You can safely dispose of the Safety Lancets, Alcohol Wipe, and Gauze Pads in your home garbage.
23. When your blood specimen has dried, close the flap of the collection card over the circles
24. Place the card in the plastic bag containing the Humidity Indicator and Desiccant Packets. Seal the bag and ensure that it is airtight.

    (Source: www.KnowAtHome.org/Espanol, accessed on 14, August 2019)

Appendix 8.9. *P. falciparum* positive sample collection plan from Adama and its surroundings (September 2019 to August 2020) and its accomplishment.

S.N	Sites	Planned	Collected			Accomplishment (%)
			Major season	Minor season	Total	
1.	Adama	70	43	28	71	100+
2.	Modjo	20	21	1	22	100+
3.	Wonji	20	18	1	19	95
4.	A. Malkasa	20	18	10	28	100++
5.	Olanciti	20	27	4	31	100++
Total		150	127	44	171	100++

Appendix 8. 10. List of publications produced during the PhD study period

1. File, T., Golassa, L., Dinka, H. *Plasmodium falciparum* clinical isolates reveal analogous circulation of 3D7 and FC27 allelic variant and multiplicity of infection in urban and rural settings. The case of Adama and its surroundings, Oromia, Ethiopia.
   Hindawi, Journal of Parasitology Research Volume 2022, Article ID 5773593, 10 pages https://doi.org/10.1155/2022/5773593.
2. File, T., Chekol, T., Solomon, G. *et al.* Detection of high frequency of MAD20 allelic variants of *Plasmodium falciparum* merozoite surface protein 1 gene from Adama and its surroundings, Oromia, Ethiopia. *Malar J* **20,** 385 (2021). https://doi.org/10.1186/s12936-021-03914-9.
3. File, T., Dinka, H. & Golassa, L. A retrospective analysis on the transmission of *Plasmodium falciparum* and *Plasmodium vivax*: the case of Adama City, East Shoa Zone, Oromia, Ethiopia. *Malar J* **18,** 193 (2019). https://doi.org/10.1186/s12936-019-2827-6.
4. Temesgen File, Hunduma Dinka. A preliminary study on urban malaria during the minor transmission season: The case of Adama City, Oromia, Ethiopia. *Parasite Epidemiology and Control*, Volume 11, 2020, e00175, ISSN 2405-6731, https://doi.org/10.1016/j.parepi.2020.e00175.
5. Temesgen File[1*], Bayissa Chala[2] Five-Year Trend Analysis of Malaria Cases in East Shawa Zone, Oromia, Ethiopia. doi: http://dx.doi.org/10.4314/ejhs.v31i1.3