Polymorphism of Block3 Region of Merozoite Surface Protein-2 Gene and Multiplicity of Infection in Plasmodium Falciparum in Urban and Rural Settings of Adama and Its Surroundings, Oromia, Ethiopia.

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Research

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Abstract

Background: Despite significant progress achieved globally in reducing malaria burden, still it is one of the major public health problems in Ethiopia. Furthermore, better understanding of genetic polymorphism of *P. falciparum*: the most virulent and predominant malaria parasite primarily targeted in malaria control and elimination program is paramount. Analysis of block 3 region of msp-2 gene of *P. falciparum* provides strong molecular evidence to evaluate the real picture of malaria epidemiology to fine-tune the ongoing control and elimination programs in the region. Thus, this study aimed to examine the status of such polymorphic gene and its implications in Adama and its surrounding.

Methods: A total of 171 Dry Blood Spot (DBS) samples were collected from uncomplicated falciparum malaria patients from September 2019 to August 2020. Tween[®] 20 and Chelex method was employed for parasite DNA extraction. A total of 148 samples were successfully amplified by nested PCR targeting msp-2 alleles (IC/3D7 and FC27) followed by gel electrophoresis for fragment analysis.

Results: Seventeen different polymorphic forms of msp-2 allelic fragments were detected in the study area. Moreover, a total of 47 (31.8%) and 41 (27.7%) were detected for IC/3D7 and FC27 allelic family, respectively. Furthermore, the multi-clonal allele type accounted for 60 (40.5%). The overall MOI was 1.4 and the expected heterozygosity is 0.49 indicating nearly intermediate malaria transmission in the study area.

Conclusions: The study revealed nearly intermediate genetic diversity and polyclonal infection of *P. falciparum* in the study area, demanding further scale up of the ongoing control and elimination efforts.

1. Background

World Health Organization (WHO) report shows that; in 2019, the number of malaria cases were 229 million, out of which 409,000 deaths was recorded. More than 90% of all malaria cases and death were only from sub-Saharan Africa (SSA) [1]. About 68% of Ethiopian population is residing in malarious area. *P. vivax* and *P. falciparum* are the two co-endemic species in the country, sharing the overall burden of 30 and 70%, respectively [2, 3]. In Ethiopia, even though malaria is responsible for a major public health problem, the various intervention efforts made so far has significantly reduced the disease burden. This successes have promoted the country to move forward and plan for malaria elimination strategies in selected areas [4]. However, the major hurdle that limited the national effort to combat malaria in Ethiopia are; the challenges related to effective implementation of major intervention strategies for malaria control like early diagnosis, prompt treatment, selective vector control, environmental management, and resource intensive nature of the programs [4, 5]. In addition, studies also show that a number of factors like, the emergence of insecticide resistance by the mosquito vectors, increased population density, global warming, poverty, the lack of effective vaccines, the emergence and spread of drug resistant strains could limit malaria control and elimination plan [6, 7]. Added to this, the frequent emergence and spread of genetic diversity of the predominant malaria parasite, *P. falciparum*, is another challenge.

.In malaria endemic region, *P. falciparum* infection is characterized by having higher genetic diversity, which is implicated in its evolutionary fitness, and consequently having more survival advantage by overcoming the control efforts [8]. Genetic diversity is also an indicator of the intensity of transmission [9], and potential deficiencies in malaria control programs [10]. The most commonly used techniques for molecular characterization of such polymorphism in malaria infection is the nested amplification of merozoite surface proteins (msp) like block 3 region of msp-2 gene [11, 20]. msp2 is a glycoprotein approximately 30 kDa encoded by the msp2 gene located on chromosome 2. It is composed by five blocks of which the central block (block 3) is the most polymorphic [12, 13]. Msp-2 is a highly polymorphic antigenic marker that has been used extensively to describe the parasite populations, thus used as a discriminatory and informative marker for strain differentiation [6]. Due to the variable non-repeat sequences as well as the varying sizes of the tandem repeats in the central region, msp2 gene is a dimorphic and existing in two main allelic families; FC27 and ICI/3D7 [14, 20]. Therefore, molecular based investigation of the diversity of *P. falciparum* and its multiplicity of infection (MOI) could be used to describe the level of malaria transmission in a given locality. For successful malaria elimination, information on the genetic profile of the parasite population in different geographical area, and factors that determine gene flow between locations is paramount. Even though, genetic diversity of *P. falciparum* has been extensively studied in different part of the world, limited and mainly a single health facility based molecular data were yet available only from, southwestern, northwestern and northeastern part of Ethiopia.

The aim of this study was, to examine genetic polymorphism of block 3 region of msp-2 gene of *P. falciparum* and its multiplicity of infection (MOI) from three districts around Adama, including Adama town administration.

2. Materials And Methods

2.1 Study sites

The study was carried out in three districts of central Ethiopia (Adama, Modjo, and Olanciti), including Adama town administration. Blood sample collection for DBS preparation and relevant patient data from malaria patient due to *P. falciparum* was collected from September 2019 to August 2020. The sites included in the study area were mainly from Adama town. The remaining were from; Adama district including Wonji located at 8km south of Adama and Awash Malkasa situated at a distance of 15km southeast of Adama 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 (Fig. 1). Adama is the major town next to the main capital in central Ethiopia, located at a distance of about 99 km southeast of Addis Ababa. Similar to the other part of the country where malaria is endemic, malaria transmission in study area 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 [15]. According to the 2007 projection of

Adama and its neighboring districts are located in the Rift Valley region of central Ethiopia (Fig. 1), where malaria is endemic. The major factors contributed for such malaria endemicity are topographic location less than 2000 meter above sea level, seasonality of rainfall pattern. In addition, average annual temperature that ranges from 16–32°C suitable for the breeding of *Anopheles arabiensis* (the predominant malaria vector in the region), and various micro ecological factors that favor mosquito breeding [15, 16].

Figure 1: Map of the study area

2.2 Sample collection and processing

A total of 171 microscopically confirmed *P. falciparum* infected patient data and blood samples were collected (aged 1 to 66) from selected health facilities in Adama, Modjo, Wonji, Awash Malkasa and Olanciti towns. Health facilities were purposively selected based on physical location and history of patient caseload. *P. falciparum* positive blood samples for Dry Blood Spot (DBS) preparation by spotting it on Whatman™ 3MM filter paper and patient data were collected from all study sites from September 2019 to August 2020. The inclusion criteria for this study was uncomplicated malaria cases due to *P. falciparum*, having history of fever onset since 24 hours of the clinical examination. For the sample and patient data collection, experienced medical laboratory technologist were selected. The recruited laboratory professionals trained to conduct finger prick and collect blood sample with patient data. For malaria microscopy, thick and thin blood films prepared, air-dried and stained with 10% Giemsa for 15 minutes. At least two independent laboratory technologist identified malaria parasite species and counted the parasitaemia level. When the sexual form (gametocytes) seen, the slide was excluded from the count. Slide considered positive after two concordant readings by two different microscopists. When discrepancy occurred, the third microscopist made the decision.

The number of parasite density per microliter (µl) of blood was calculated by using the following formula:

Parasite Density per microliter (µl) of blood = Number of Parasite counted $\times \left(\frac{8000}{200} \right)$, [17, 18]. Assuming

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

For comparison with ranked order variables during data analysis, parasitemia were categorized in to five levels: L1 (< 50 parasite/µl blood), L2 (50–499 parasite / µl blood), L3 (500–4999 parasite / µl blood), L4 (5000–49999 parasite/ µl blood), and L5 ($\geq 50,000$ parasite/ µl blood) [19].

2.3 Extraction of *P. falciparum* DNA and amplification of msp-2 gene

The parasite genomic DNA extraction and its msp-2 gene amplification was conducted at malaria research laboratory, Akililu 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 [20].

The polymorphic region of *P. falciparum* msp-2 gene (block 3) was used as a genetic marker for the genotyping of parasite populations.

Nested PCR of msp-2 (block 3) polymorphic region was performed by slight modification of primers and methods from the previously described reports [20, 21] (S1-Table). In brief, initial amplification (N1) of msp-2 gene were carried out in a final volume of 20µl amplification mixture containing 0.5 µl of each primer, 5x FIREPol® .Master Mix (MM), 11 µl of nuclease free water aliquot to 16µl to which 4µl of DNA template was added. In the second (N2) amplification reaction, 2µl of the amplicon product was used. The PCR amplification profile for both N1 and N2 reactions includes; initial denaturation at 95°C for 3 minutes, denaturation at 94°C for 1 minute, annealing 58°C for 1 minute, elongation at 72°C for 2 minutes and final elongation for 5minutes. Allele specific positive control 3D7 and DNA free negative control were used in each set of the reactions as described elsewhere. Gel-electrophoresis of DNA fragment for 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) (S2-Figure). 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 [22]. When the size of allele fragment was found less than 20 bp interval they were considered the same [23].

2.4 Data Analysis

Descriptive statistics was used to calculate the frequency of each msp-2 allelic families in relation to the total number of gene successfully amplified for that locus. Multiplicity of Infection (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-2 gene. When alleles in each family was less than 20bp, they 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. All statistical tests were performed by using SPSS version 20.0 (SPSS Inc., Chicago, USA).

The expected heterogeneity (He) was calculated by the formula;

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

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

2.5 Ethical Consideration

Ethical approval

of the study obtained from Institutional Ethical Review Board of Adama Science and Technology University (ASTU). Oromia Regional State Health Bureau, approved and gave consent for the study protocol. In addition, written informed consent obtained from the patients or their parents/guardian prior to recruitment.

3. Results

3.1 Demographic and parasitological data

From 171 microscopically confirmed malaria patients due to *P. falciparum*, 148 samples successfully amplified for msp-2 gene. Of which 105 (71%) were males. The age of the study participant ranged from 1 to 66. Mean $\pm$ SD ($27.0 \pm 13.6^*$) years. Asexual parasite density ranged from 64–104,320 parasites/ μ l with a geometric mean of 5,654 parasites/ μ l. Of all study participants 85 (57%) were from urban inhabitants. Of all the study subjects by occupation, students, daily laborers and farmers alone accounted for 113 (76%) *P. falciparum* malaria cases in the study area. Moreover, the incidence of *P. falciparum* isolates were having significant relation ($\chi^2 = 0.017$) with occupation type (Table 1).

Table 1

Socio-demographic characteristics and parasitological data of symptomatic malaria patients due to *P. falciparum* clinical isolates genotyped for msp-2 gene from Adama and its surroundings (n = 148).

Patient characteristics	Sample genotyped	Chi square (χ^2)
Mean age (year)	27.0 $\pm$ 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
Occupation		
Farmer	31(21%)	0.017
Housewife	12(%)	
Daily laborer	35(23.8%)	
Government employee	13(8.8%)	
NGO employee	2(1.3%)	
Business man	7(4.7%)	
Student	47(32%)	
Geometric mean of parasitic density (P/ μ l) of blood	5,654	
Parasite density range (P/ μ l) of blood	64–104,320	
Parasitaemia level	5 (3.3%)	0.075
- (50–499 P/ μ l of blood)		
- (500–4999 P/ μ l of blood)	63(42%)	
- (5000-49,999 P/ μ l of blood)	75(50.6%)	
- ($\geq$ 50,000 P/ μ l of blood)	5(3.3%)	

3.2 Allele frequency, genetic diversity and multiplicity of infection

Allele genotyping of msp-2 gene of *P. falciparum* in Adama and its surroundings revealed its polymorphic nature. The frequency of IC/3D7 and FC27 was 31.8% (47/148) and 27.7% (41/148), respectively. Moreover, 40.5% (60/148) carried both IC/3D7 and FC27 alleles, with the overall MOI of 1.4 and the expected heterozygosity (H_e) of 0.49.

In addition, size polymorphism demonstrated in the study revealed that 106 and 99 samples were successfully genotyped for IC/3D7 and FC27 variant forms, respectively. In this study among the msp-2 isolates: we observed seven IC/3D7 (200-700bp) and ten FC27 (250-700bp) different size polymorphic alleles (Table 2).

Table 2

Genetic diversity, allelic frequency and allelic fragment size of block 3 region of msp-2 gene isolated from symptomatic patients in Adama and its surroundings, Oromia, Ethiopia (n = 148).

Msp-2 alleles (n = 148)	Allele frequency n (%)	Fragment size (bp)	Number of alleles	Overall MOI	Expected Heterozygosity (<i>H_e</i>)
IC/3D7	47 (31.8)	200–700	7	1.4	0.49
FC27	41 (27.7)	250–700	10		
3D7 + IC/3D7	60 (40.5)				
Total	148				

Comparison of geometric mean of the parasite density at different age group shown disproportionate burden of parasitemia in school age children (SAC). The overall rate of clinical prevalence of msp-2 gene allelic variant among symptomatic patients tends to increase with age groups, although the variation was not statistically significant ($\chi^2 = 0.09$). Likewise, though not linearly MOI slightly risen up with age group (Table 3).

Table 3

Distribution of msp-2 allelic variants, average MOI, and geometric mean of parasite density among different age groups of *P. falciparum* infection in Adama and its surroundings.

Characteristics	Age groups in year				
	< 5 n (%)	5–14 n (%)	15–24 n (%)	> 24 n (%)	Total n (%)
ICI/3D7	2 (4.2)	4 (8.5)	10 (21)	31 (66)	47
FC27	2 (4.8)	7 (17)	14 (34)	18 (44)	41
ICI/3D7 + FC27	1 (1.7)	6 (10)	28 (47)	25 (41.7)	60
Total	5 (3.4)	17 (11)	52 (35)	74 (50)	148
Average MOI	1.25	1.54	2.1	1.5	
Geometric mean of parasite density	6455	7419	4324	6381	

Analysis of the urban rural distribution of msp-2 allelic families in Adama and its surrounding shown no significant variation of allelic variants in urban and rural localities ($P = 0.56$). However, as revealed in

(Fig. 2) msp-2 allelic family in study area revealed similarity, except higher MOI in school age children from urban localities compared with similar age groups from rural area.

Figure 2. Urban rural features in MOI of *P. falciparum* msp-2 allelic families isolated from different age groups of symptomatic patients in Adama and its surroundings, Oromia, Ethiopia (n = 148).

On the other hand, the distribution of msp-2 allelic variant in different study sample collection sites indicated a highly significant variation ($p = 0.00$) (Fig. 3).

Figure 3. The distribution of *P. falciparum* msp-2 allelic families isolated from different sample collection sites from symptomatic patients in Adama and its surroundings, Oromia, Ethiopia (n = 148).

Moreover, the distribution of msp-2 allelic variants detected in the study area showed slight variation during major malaria season and relatively stable during minor malaria season (Fig. 4).

Figure 4. The distribution of *P. falciparum* msp-2 allelic families clinically isolated during major and minor malaria season from symptomatic patients in Adama and its surroundings, Oromia, Ethiopia (n = 148).

4. 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 strategy is improving malaria surveillance and response [24]. In this regards, molecular epidemiological study approach like characterization of block 3 region of *P. falciparum* msp-2 provides comprehensive molecular evidence for effective disease surveillance that ultimately transformed to core interventions to the control and elimination of malaria.

The present study revealed that consistence with our previous report [25] and that of [15], incidence of *P. falciparum* isolates was higher in male (71%) individuals (Table 1). The major factors that may account for such higher malaria cases compared to female is that older boys and men may be at special risk for malaria from occupational and travel-related factors [24]. In addition, consistent with the report of [26] incidence of *P. falciparum* isolates in the present study was significantly related to occupation type ($\chi^2 = 0.017$) (Table 1). This could be due to strong relation of malaria incidence with lower standard of living. This might have contributed for the occurrence of 76% of all *P. falciparum* isolates in the present study only from farmers, daily laborers, and students alone.

Investigation of msp-2 block-3 region of *P. falciparum* genetic profile revealed in this study was the first in its kind in our study area. Moreover, we examined the seasonal and spatial distribution of msp-2 allelic variants in selected sites within the designated study area, together with their urban rural counterpart. Of the total successfully genotyped msp-2 gene the monoclonal alleles of IC/3D7 and FC27 constitute 31.8% and 27.7% respectively (Table 2). Report from maritime region of Togo [27] and Ponte-Noire, Republic of Congo) [28] complement our finding. Moreover, the number of msp-2 genotypes detected for

IC/3D7 and FC27 was 7 and 10 respectively (Table 2). Although the number of genotypes might have been underestimated due to the limitation of the techniques. Fragment size polymorphism described in this study is nearly comparable with the previous report from Republic of Congo [28], Nigeria [29], Sudan [17], and northeastern Ethiopia [30]. Other reports from Congo Brazzaville [31], north western Ethiopia [32] shown the predominance of IC/3D7 allelic family. Such inconsistency in *P. falciparum* allelic size polymorphism could be due to geographical location, transmission intensity and scope of sample population covered in the study. The rate of msp-2 polyclonal infection identified was 40.5%, with the overall MOI of 1.4 (Table 2). This finding is lower than the previous report from southwestern Ethiopia [33] northwestern Ethiopia [32], Sudan [17], Cameroon [34], and Nigeria [35]. And somewhat higher than the previous report from north eastern Ethiopia [30], and Ghana [36]. 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 [37]. The overall MOI identified in our study area could serve as proxy of transmission intensity for targeted intervention in the region. Moreover, in the present study we investigated the expected heterozygosity (He) of 0.49 (Table 2) that nearly indicates an intermediate transmission pattern in the study area.

In this study, analysis of the variation of msp-2 allelic frequency across different age groups generally tends to rise up with age group in parallel with *P. falciparum* clinical prevalence during the study period. However, the variation was not statistically significant ($P = 0.09$) (Table 3). The general increment observed in this study is in agreement with the previous five year retrospective study reported in Adama, where the clinical cases of *P. falciparum* rose up with age group [16]. In the present study, we found that MOI tends to increase with age groups until the age of late adolescent (Table 3). This finding differs from the report from hyper endemic area of Burkina Faso [38], where they reported the existence of negative relationship between MOI with patient age. On the other hand, consistent with our finding other report from Burkina Faso [37], and Tanzania [39], explained that episode of infection in children is commonly for very short duration and the duration of episode of infection increases with age contributing to the rise in MOI in other age groups. When the correlation of msp-2 allelic variant and parasitemia level was examined, we found no statistically significant correlation (Pearson correlation = -0.19, $X^2 = 0.07$) existed between msp-2 allelic variants with parasitemia level in different age groups (Table 3). This could be due to the reduced transmission intensity of *P. falciparum* infection in the study area. Even though different factors may contribute to the fluctuation of parasitaemia level in symptomatic patients over time, the highest geometric mean of microscopically detected parasitemia level was in school age children (5–14 years old) (Table 3). This was due to delayed acquisition of protective immunity of this age groups [40].

Unlike the report from Burkina Faso, where there was a difference in some allelic family observed in rural and urban settings probably due to urbanization [37], in the present study, we observed no statistically significant variation in both rural and urban localities ($P = 0.56$). This indicates the existence of similar malaria epidemiology in both rural and urban settings of the study area. Slightly higher MOI detected school age children (Fig. 2) could be due to higher (70%) malaria case due to *P. falciparum* of this age group from urban area. Moreover, study site based distribution of msp-2 allelic variant (Fig. 3) showed a

highly significant variation ($P = 0.000$). This could be largely due to the differences in local micro-ecological factors that may result in varying mosquito population density, change in parasite vector interaction, change in host immunity induced by parasite interaction, and spatial heterogeneity of the study sites under consideration [41, 42]. These major factors might have affected local transmission pattern contributing to such variation. In Ethiopia there are two malaria season. The major season follow the rainy summer season from June to August that begins from September to December. The minor malaria season follow the shorter rainy season around April and May (FMOH, 2015). Analysis of intra seasonal variation on the distribution of msp-2 allelic variant (Fig. 4) showed relatively higher variation during major malaria season, although the variation is not statistically significant ($p = 0.9$). In complement with this finding [37] reported that the dominance of any msp-2 allele was dependent on transmission intensity and independent of seasonal change.

5. Conclusions

The present molecular study revealed nearly moderate transmission of *P. falciparum* clinical isolates among symptomatic malaria patients: having polyclonal infection rate of (40.5), MOI (1.4) and *He* (0.49) in such malaria endemic region of central Ethiopia. Therefore, to sustain such declining phase of *malaria* transmission, in addition to the classical epidemiological study approach: the ongoing malaria control and elimination program should be accompanied with similar molecular surveillance for targeted intervention. Moreover, the present study revealed absence of significant variation in allele frequency in both urban and its rural counterpart and similar allele frequency during the major and minor malaria season, reflecting frequent inbreeding among the existing parasite strains in both settings. This implicates the presence of similar malaria epidemiology demanding similar intervention in both urban and rural areas during the major and minor malaria season in the study area. Thus, the findings described in this 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.

Abbreviations

Bp: Base pair; msp-1: Merozoite surface protein-2; MOI: Multiplicity of infection; PCR: Polymerase chain reaction; SAC: School age children.

Declarations

Ethics approval and consent to participate

The research and ethical committee of Adama Science and Technology University reviewed and approved the study protocol, as verified through certificate reference number RECSOANS/BIO/01/2019. Oromia Health Bureau also approved the study protocol.

Consent for publication

Loading [MathJax]/jax/output/CommonHTML/jax.js

- Not applicable

Availability of data and materials

All relevant data is included in manuscript, and the datasets analyzed in the study are available from the corresponding author on reasonable request. Additional data uploaded with main document.

Competing interests

- The authors declare that they have no competing of interest

Funding

- No funding was obtained for this study

Authors' contributions

TF designed, conducted and analyzed the study, drafted and wrote the manuscript. HD designed the study and finally reviewed and approved the manuscript. LG conceived the idea, designed the study, supervised the molecular laboratory work, and reviewed and approved the manuscript.

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Figures

Figure 1

Map of the study area

Figure 2

Urban rural features in MOI of *P. falciparum* msp-2 allelic families isolated from different age groups of symptomatic patients in Adama and its surroundings, Oromia, Ethiopia (n = 148).

Figure 3

The distribution of *P. falciparum* msp-2 allelic families isolated from different sample collection sites in Adama and its surroundings, Oromia, Ethiopia (n = 148).

Figure 4

The distribution of *P. falciparum* msp-2 allelic families clinically isolated during major and minor malaria season from symptomatic patients in Adama and its surroundings, Oromia, Ethiopia (n = 148).

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