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Low genetic diversity of *Plasmodium falciparum* merozoite surface protein 1 and 2 and multiplicity of infections in western Ethiopia following effective malaria interventions

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Background: Genetic diversity of malaria parasites can inform the intensity of transmission and poses a major threat to malaria control and elimination interventions. Characterization of the genetic diversity would provide essential information about the ongoing control efforts. This study aimed to explore allelic polymorphism of merozoite surface protein 1 (*msp1*) and merozoite surface protein 2 (*msp2*) to determine the genetic diversity and multiplicity of *P. falciparum* infections circulating in high and low transmission sites in western Ethiopia.

Method: Parasite genomic DNA was extracted from a total of 225 dried blood spot collected from confirmed uncomplicated *P. falciparum* malaria infected patients in western Ethiopia. Of these, (163) 72.4% and (62) 27.6 % of the samples were collected in high and low transmission areas, respectively. Polymorphic *msp1* and *msp2* genes were used to explore the genetic diversity and multiplicity of falciparum malaria infections. *Msp1* genotyping was successful in 141/163 and 55/62 samples collected from high and low transmission areas, respectively. On the other hand, genotyping of *msp2* was carried out among 85.3 % and 96.8 % of the samples collected in high and low transmission sites, respectively. *P. falciparum msp1* and *msp2* genes were amplified by nested PCR and the PCR products were analyzed by Qiaxcel software. A p-value of less or equal to 0.05 was considered significant.

Result: High prevalence of falciparum malaria was identified in children less than 15 years as compared with those ≥ 15 years old (AOR= 2.438, P=0.005). The three allelic families of *msp1* (K1, MAD20 and RO33) and the two allelic families of *msp2* (FC27 and 3D7), were observed in samples collected in high and low transmission areas. However, MAD 20 and FC 27 alleles were the predominant allelic families in both setting. *P. falciparum* isolates circulating in western Ethiopia had low genetic diversity and mean MOI. No difference in mean MOI between high transmission site (mean MOI 1.104) compared with low transmission area (mean MOI 1.08) ($p > 0.05$). Expected Heterozygosity of *msp1* gene was slightly higher in isolates collected from high transmission site ($H_e = 0.17$) than those isolates from low transmission ($H_e = 0.12$). However, the heterozygosity of *msp2* gene was not different in both settings (P_{fmsp2} : 0.04 in high transmission; p_{fmsp2} : 0.03 in low transmission).

Conclusion: *P. falciparum* from clinical malaria cases in western Ethiopia have low genetic diversity and multiplicity of infection irrespective of the intensity of transmission at the site of sampling. These may be signalling the effectiveness of malaria control strategies in Ethiopia;

although further studies are required to determine how specific intervention strategies and other parameters that drive the pattern.

Key words: MOI / genetic diversity/ *P. falciparum*/ malaria transmission/ western Ethiopia

Background

Malaria remains a global public health problem with 229 million cases and 409 000 deaths globally in 2019. *Plasmodium falciparum* remains the most dangerous malaria parasite species to humans with important public health consequences mostly in Africa (1).

Multiple *Plasmodium* strains (also known as multiple infections) is common in malaria-endemic areas, which may result from the bite of mosquitoes infected with more than one clone or from multiple bites (2). Interaction and competition of different strains for resources within a host during their life cycles may be important for survival and promotes diversity of the parasite (3, 4). The presence of multiple infecting strains may also affect key phenotypic traits, including drug resistance (5, 6) and risk of severe disease (7, 8). Characterizing genetic diversity of malaria in infected individuals and populations of different endemic settings could be helpful in determining the effectiveness of malaria interventions. Multiplicity of the parasite has been proposed as a marker to measure changes in transmission intensity in time and space (9, 10).

Malaria parasites genomic epidemiology studies have been assessed using multiplicity of infection (MOI) and the proportion of polyclonal infections (also termed ‘multiple clone infections’ (9, 11). It is generally assumed that MOI is higher in high transmission settings, while in low transmission settings, most individuals might carry single clone infections. A correlation between transmission intensity and MOI has been reported (12-14). But, a study done in Eswatini showed absence of association between multiplicity of infection and transmission intensity, most likely due to infections being imported (15). In Ethiopia, altitude, and climate (rainfall and temperature) are the most significant determinants for malaria transmission. *P. falciparum* is the dominant malaria parasite, accounting for 60 to 70% of all malaria cases (16). Over the last two decades, malaria morbidity and mortality have been reduced dramatically in the country following intensive use of insecticide residual spray, long-lasting insecticide treated

nets, chemotherapies, improved diagnosis and case management in recent years (17). Towards local strategies for malaria elimination; it is important to assess genetic diversity of malaria parasite in different settings to determine the effectiveness of the ongoing interventions and to better understand parasite population circulating in a given geographic area. In this study we focus on Western Ethiopia, where there is relatively limited information about the genetic diversity and MOI of *P. falciparum* infections. The aim of this study was to compare genetic diversity of *P. falciparum* isolates collected from high and low transmission sites, western Ethiopia.

Methods

Study site and period

This study was carried out during high malaria transmission season from September to December 2020 in four health centers located in areas with different levels of malaria transmission in western part of Ethiopia. These included Sherkole and Horazhab health centers which are found in Assosa zone in the Benishangul-Gumuz Region of Ethiopia. Both health centers are located in areas along the western borders with Sudan which has been characterized with the highest transmission intensity (18). Malaria transmission remains generally high in the western Ethiopian border area near Sudan (19). Sherkole health centre is located in Sherkole district which is bordered by Sudan in the north. Sherkole town is about 754 km from Addis Ababa. Horazhab health center is located in Kurmuk district, and it is bordered by Sudan in the north and west. It is about 769 km from Addis Ababa (figure 1).

Anger Gute and warabo health centers are found in Anger Gute town, Gida Ayana district, East Wollega Zone, Oromia Regional State. Anger Gute town is 360 Km away from Addis Ababa. Malaria transmission in Anger Gute area is low and stable. *P. falciparum* infection prevalence among children 2-10 years was <5% (18). Incidence of malaria in and around Anger Gute town was 3.43 per 1000 population at risk of the disease and the malaria trend between 2014 to 2018 indicated nearly unchanged numbers of malaria cases (20).

Figure 1. Map of Ethiopia showing the study area, Gida Ayana, Sherkole and Kurmuk districts, Western Ethiopia.

Study design

A health facility based cross-sectional study was conducted from September to December, 2020.

Consented febrile patients seeking malaria diagnosis at the study sites were recruited.

Sample size

The sample size was determined using a single population formula, using 13.1% prevalence of malaria in Benishangul-Gumuz regional state(16), a 95% confidence level and 5% precision. Accordingly, the calculated sample size was 175. With 13% adjustment for non-response rate, a

total of 198 uncomplicated falciparum malaria patients were included in the study. The number of patients included from high and low transmission sites were proportional to the number of confirmed *P. falciparum* reported from the study health facilities in 2019 G.C. Accordingly, from a total of 230 samples, 166 and 64 were collected from high and low transmission areas, respectively.

Study population

At each health center, consenting patients with uncomplicated malaria whose age was greater than 6 months, and microscopically-confirmed to be infected with *P. falciparum*, were enrolled. For confirmatory diagnosis of *P. falciparum*, finger-prick blood samples were used for thick and thin blood films for slide microscopy were made. Each slide was stained with 10% Giemsa for 10 min and 100 fields examined before designating a sample negative(21). Once the patients were microscopically confirmed of *P. falciparum* infection, they were consented to give finger-prick blood samples to prepare dried blood spots (DBS) on filter paper (Whatman No. 1001 320, International Ltd. Maidstone, England). The DBS were kept in plastic bags with desiccants until molecular analysis. Patients with mixed infections with other *Plasmodium* species were excluded from the study.

Molecular genotyping

Molecular detection of parasite genomic DNA was done at Medical Research Council Unit The Gambia at the London School of Hygiene & Tropical Medicine. DNA extraction was done from DBS using the Chelex protocol as earlier described (22). *P. falciparum* detection was performed by Var gene acidic terminal sequence (varATS) real-time PCR as previously described (23). For genetic diversity, genomic DNA of the parasite was amplified by multiplex primary and Nested PCR using allele specific Primers (Table 1) of *msp1* and *msp2* as per published protocol (24). Allelic variants of *msp1* (K1, MAD 20 and RO33) and *msp2* (FC27 and 3D7) were detected by allelic family-specific nested PCR amplification. Genotypes were distinguished using their fluorescent dye (indicating the allelic family) and by their size which was determined by Qiaxcel software.

Data analysis

PCR amplicon bands for *msp1* and *msp2* were sized and binned using the Qiaxcel analysis software. SPSS 20.0 statistical software package (SPSS, Inc, Chicago, USA) was used for statistical analysis. The allelic frequency of *msp1* or *msp2* was determined by counting the number of samples observed with a particular allelic variant, divided by the total number of allelic variants observed in all the samples analyzed for *msp1* or *msp2*.

An infection with a single allele for both targets was considered as a monoclonal infection and those infections with more than one PCR amplicon for either or both targets were considered as polyclonal. Mean MOI in both settings of malaria transmission were determined as the ratio of the total number of *P. falciparum* genotypes detected in *msp1* and *msp2* by the number of samples positive for *msp1* and *msp2*. Student's t test was used to compare the mean MOI of both loci between high and low transmission sites.

Expected heterozygosity (HE), a measure for genetic diversity, was calculated by using the following formula: $HE = [n/(n - 1)][(1 - \sum p_i^2)]$, where *n* is the number of isolates sampled and *p_i* is the allele frequency at a given locus. A p-value of less or equal to 0.05 was considered suggestive of a statistically significant difference between sites.

Ethical consideration

Ethical clearance was obtained from the Ethiopian National Ethic Review Committee and Addis Ababa University, Aklilu Lemma Institute of Pathobiology, IRB. Permission to conduct the study at the health facilities was sought from the relevant regional and district health authorities. Written informed consent was obtained from adult study participants and a parent or guardian of a child. Written informed assent was also taken from children.

Results

Out of 230 microscopically confirmed *P. falciparum* cases enrolled in the study, 225 of them tested positive by PCR. 72.4% (163/225) and 27.6 % (62/225) PCR positive samples were collected from high and low malaria transmission sites, respectively. The mean age of study participants was 17.8 ± 12.7 years and with an age range of 7 months – 75 years old. Ratio of male to female was 1.27: 1. The prevalence of falciparum malaria was higher in children less than 15 years as compared with those ≥ 15 years old (AOR= 2.438, P=0.005). No significant difference in *P. falciparum* infections by gender was observed ($p > 0.05$), (Table 1).

Table 1: Sociodemographic characteristics and occurrence of malaria in western Ethiopia

Variables		High transmission	Low transmission	COR	P-value	AOR	P-value
Sex	Male	85	41	1.79	0.061	1.827	0.057
	Female	78	21	1		1	
Age categories							
< 15 years		90	21	2.41	0.005	2.438	0.005
≥ 15 years		73	41	1		1	

Genetic polymorphism of *msp1* and *msp2* genes

Msp1 genotyping was successful in 87% (141/163) and 88.7% (55/62) of samples collected from high and low transmission areas, respectively. Similarly, for *msp2* genotyping, 85.3 % (139/163) and 96.8 % (60/62) samples were successfully genotyped from high and low transmission sites, respectively. For *msp1*, the MAD20 allele was dominant, with a total of 87%, 25.5% and 5 % of the samples from high transmission site had MAD20, K1 and RO33 allelic families of *msp1*, respectively. Similarly, 93%, 14.5% and 5% samples collected from low malaria transmission setting had MAD20, K1 and RO33 *msp1* allelic families, respectively. Multiclonal infections with K1+MAD20, MAD20+R033 combinations were also observed. In high transmission

setting, K1+MAD20 accounted for 14.9% while the frequency of this allelic combination was 10.9% in low transmission setting (Figure 2). Allelic combinations of K1 and RO33 and K1/MAD20/RO33 were not detected in any infection.

Figure 2: Distribution of *msp-1* allele types among *P. falciparum* isolates in different level of malaria transmission, western Ethiopia. HMT: high malaria transmission, LMT: low malaria transmission.

Both FC27 and 3D7 *msp2* allelic types were identified among the samples collected from both study sites. However, FC27 was the frequent allelic type in western Ethiopia. There was no difference in the frequency of this allele in high transmission (94.96%) and low transmission (95%) areas. Few multi-allelic infections combining 3D7 +FC27 families were detected (Figure 3).

Figure 3: Distribution of *msp2* Allelotypes by intensity of malaria transmission in western Ethiopia.

In western Ethiopia, monoclonal *P. falciparum* infections were predominant, representing 82.3% (116/141) and 87.3 % (48/55) of the samples genotyped for *msp1* in high and low transmission sites, respectively. Similarly, 97.2 % (135/139) and 96.7 % (58/60) of isolates from high and low transmission areas were monoclonal *msp2*, respectively. No significant difference was observed in the proportion of *msp1* or *msp2* multiplicity of infection with sex, age group of study participants and intensity of transmission ($P > 0.05$) (Table 2).

Table 2: Proportion of multiplicity of *P. falciparum* infection in relation to sociodemographic indices of patients and level of malaria transmission, western Ethiopia

Variables	<i>msp1</i> (n=196)		AOR	P-value	<i>msp2</i> (n=199)		AOR	P-value
	monoclonal	polyclonal			monoclonal	polyclonal		
Sex								
Male	95	15	0.67	0.31	107	4	1.61	0.6
Female	69	17			86	2		
Age categories								
< 15 years	82	15	1.2	0.64	134	3	0.95	0.931
≥15 years	82	17	1		58	3	1	
Level of transmission								
High	116	25	0.69	0.43	135	4	1.11	0.91
Low	48	7	1		58	2	1	

P. falciparum isolates collected from western Ethiopia had low mean MOI and there was no significant difference in mean MOI between high transmission (mean MOI=1.104) and low transmission (mean MOI=1.08) ($p > 0.05$). Expected Heterozygosity of *msp1* gene was slightly higher in high malaria intensity sites ($H_e = 0.17$) than isolates from low malaria intensity ($H_e = 0.12$). On the other hand, heterozygosity of *msp2* gene was not different in both settings ($P_{fmsp2} = 0.04$; $p_{fmsp2} = 0.03$), (Table 3).

Table 3: MOI and heterozygosity of *msp1* and *msp2* of *P. falciparum* in western Ethiopia

Gene/ genes	high transmission(n=280)	Low transmission(n=115)	p- value
mean MOI	1.104	1.08	0.44
<i>msp1</i> He	0.17	0.12	
<i>msp2</i> He	0.04	0.03	

Discussion

Measuring the complexity of infection and genetic diversity of parasite populations across different endemicities may be used as an indicator to evaluate the efficacy of ongoing control and elimination strategies. While a few studies have investigated MOI and genetic diversity of *P. falciparum* population circulating in western Ethiopia, these didn't represent populations relatively high and low transmission sites as presented here. The prevalence of uncomplicated falciparum malaria in the populations studied was higher in children ≤ 15 years than those ≥ 15 years old (AOR= 2.438, P=0.005) and this was in agreement with previous reports from Ethiopia and Kenya (25, 26). Immunity to malaria by an individual depends on their age, the number of infectious mosquito bites (27) and by the intensity of transmission(28, 29). In high transmission areas, burden of malaria is mainly among young children unlike in areas with low transmission, where the population has low exposure to infection and malaria occurs in all age groups ((25, 30, 31).

The three allelic families of *msp1*(K1, MAD20 and RO33) and FC27 and 3D7 of *msp2* allelic families were identified in the study areas. However, MAD 20 of *msp1* and FC27 of *msp2* allelic families were the predominant alleles in both areas with variable malaria endemicity. There was no difference in the frequency of *msp1* or *msp2* allelic families by intensity of transmission. Therefore, population composition of *P. falciparum* isolates in western Ethiopia shows monoclonality infections regardless of the difference in intensity of transmission intensity and geographic separation of the parasites. This finding suggests the likely presence of high level of inbreeding among the circulating clones between the study sites. Previous reports had also indicated that malaria parasites in Ethiopia have moderate levels of genetic diversity and similar

population structure of the parasite (32). They presented the lowest levels of heterozygosity in a continent-wide *P. falciparum* genomic analysis(33), indicating the need to further determine how they have evolved and are responding to the general interventions recommended for all malaria populations.

The predominance of the MAD 20 for *msp1* and the FC27 allelic family for *msp2* among *P. falciparum* isolates were inconsistent with previous studies in other parts of Africa; North Central Nigeria (34), Equatorial Guinea (35), and Northwest Ethiopia (36). Although this corroborates previous studies from Ethiopia finding similar patterns with the predominance of MAD 20 (37) and FC27 (38, 39), other reports had shown the predominance of K1 of *msp1* and 3D7/IC1 of *msp2* in southwestern Ethiopia (40). This is either due to variance between populations or changes that may have occurred over time following selection by pressure from interventions and reducing transmission intensity.

Most of the participants had monoclonal infections at both *msp1* or *msp2* loci in both settings. As with previous reports, multiplicity of infection (MOI) was also not associated with age groups (34, 38). This was in contrast to other studies that found a correlation between polyclonal infection with age groups (41), though this was in a high transmission region in Burkina-Faso in west Africa. MOI was not associated with sex of the patients as report from Burkina Faso (42). Overall, *P. falciparum* isolates from western Ethiopia showed low mean MOI and limited genetic diversity, with no differences based on intensity of transmission, a pattern that has been seen in some malaria endemic regions of Africa, the prevalence of infections have generally become low and imported cases are common (15, 43, 44). Examples of these include Senegal and Eswatini, which are now heading towards pre-elimination. Though high MOI, and genetic diversity has been associated with higher malaria-endemicity, these indices as determined by *msp1* and *msp2* typing may not be sensitive enough at a lower overall transmission level as seen in Ethiopia (12, 35, 45, 46). However, the lack of differences with the relative variance in transmission intensities might be an indication that malaria control measures that reduce entomological inoculation rate (47, 48), and deployment of artemisinin-combination therapy that reduce population diversity by removing the drug sensitive parasites(49) have been effective in Ethiopia. It is also possible differences in human demography, ecology and *Anopheles* mosquito vector might be shaping *P. falciparum* population structure in malaria endemic sites (50-52).

Within-host competitive interactions of *P. falciparum* may also determine diversity of the parasite (3). Malaria transmission in Ethiopia is driven mainly by *Anopheles arabiensis* while *An. pharoensis*, *An. funestus* and *An. nili* are secondary vectors(53, 54), unlike the mostly *An gambiae* seen across the most endemic regions of west and central Africa.

Conclusion: *P. falciparum* isolates were mainly monoclonal with low MOI and lesser genetic diversity. Mean MOI and diversity of the parasite were not associated with intensity of malaria transmission. Hence, MOI and genetic diversity of *P. falciparum* might not be a good predictor of transmission intensity in all malaria endemic areas. There is a need to find out an inclusive measurement of intensity of transmission in all malaria endemic areas. The observation of low genetic diversity and multiplicity of infections in the study area may signal the effectiveness of the recent deployment of massive malaria control strategies in Ethiopia; however, this needs further studies to map the different intervention strategies and other parameters that could affect parasite genetic diversity.

Abbreviations: DBS: dried blood spots; IRB: Institute Review Board; PCR: polymerase chain reaction; varATS: Var gene acidic terminal sequence; MOI: multiplicity of infection; *mssl*: merozoite surface protein 1; *mss2*: merozoite surface.

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Authors' contributions

Conceptualization: Dr. Lemu Golassa and Geletta Tadele

Supervision of the overall work: Dr. Lemu Golassa, Dr. Alfred Amambua-Ngwa and Dr. Sisay Dugassa

Methodology: Geletta Tadele and Dr. Lemu Golassa

Laboratory work: Geletta Tadele, Fatou K. Jaiteh, Eniyou Oriero and Mary Oboh

write up of the original draft of the manuscript: Geletta Tadele

Reviewing and Editing the manuscript: Dr. Lemu Golassa, Dr. Alfred Amambua-Ngwa , Dr. Sisay Dugassa , Eniyu Oriero and Mary Oboh

All authors read and approved the final manuscript.

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Availability of data and materials

The datasets and analyzed result of the study are available on the corresponding author and can be obtained on reasonable request.

Ethics approval and consent to participate

Ethical approval was obtained from the Ethiopian National Ethic review committee, Addis Ababa, Ethiopia (No. MoSHE 04/246/66) and Aklilu Lemma Institute of Pathobiology, Institutional review board, Addis Ababa University (No. ALIPB IRB/ 19/2012/20). Written informed consent was obtained from adult study participants and a parent or guardian of a child. Written informed assent was also taken from children.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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