Genetic diversity and genotype multiplicity of *Plasmodium falciparum* infection in patients with uncomplicated malaria in Chewaka district, Ethiopia

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Abstract

Background: Genetic diversity in *Plasmodium falciparum* poses a major threat to malaria control and elimination interventions. Characterization of the genetic diversity of *Plasmodium falciparum* strains can be used to assess intensity of parasite transmission and identify potential deficiencies in malaria control programmes, which provides vital information to evaluating malaria elimination efforts. In this study, we investigated the *P. falciparum* genetic diversity and genotype multiplicity of infection in parasite isolates from cases with uncomplicated *P. falciparum* malaria in Southwest Ethiopia.

Methods: A total of 80 *P. falciparum* microscopy and qPCR positive blood samples were collected from study participants aged six months to sixty years, who visited the health facilities during the evaluation of a therapeutic efficacy study of arthemeter-lumefantrine from September-December, 2017. Polymorphic regions of the *msp-1* and *msp-2* were genotyped by nested polymerase chain reactions (nPCR) followed by gel electrophoresis for fragment analysis.

Results: Of 80 qPCR-positive samples analyzed for polymorphisms on *msp-1* and *msp-2* genes, the efficiency of *msp-1* and *msp-2* gene amplification reactions with family-specific primers were 95 % and 98.8%, respectively. A total of 29 *msp* alleles (10 for *msp-1*and 19 for *msp-2*) were detected. In *msp-1*, *K1* was the predominant allelic family detected in 47.7% (42/88) of the samples followed by *Mad20* and *RO33*. For *msp-2*, the frequency of *FC27* and *IC/3D7* were 77% (57/74) and 76% (56/74), respectively. Eighty percent (80%) of isolates had multiple genotypes and the overall mean multiplicity of infection was 3.2 (95% CI: 2.87- 3.46). The heterozygosity index was 0.43, and 0.85 for *msp-1* and *msp-2*, respectively. There was no significant association between multiplicity of infection and age or parasite density.

Conclusions: The study revealed high levels of genetic diversity and mixed-strain infections of *P. falciparum* populations in Chewaka district, Ethiopia; reflecting both the endemicity level and malaria transmission remained high and more strengthened control efforts are needed in Ethiopia.

Background

The intensification of malaria control interventions has resulted in its global decline, but it remains a significant public health burden across several malaria-endemic countries [1]. Ethiopia is also one of the most malaria epidemic-prone countries in Africa [2]. The trends in malaria over the past five years have also shown a decline in malaria cases and reduced epidemics [3]. The key interventions which have been contributing to such significant decline includes; introduction of prompt and effective treatment with artemisinin-based combination therapy (ACT) to treat uncomplicated *Plasmodium falciparum* malaria, the distribution of long-lasting insecticidal nets (LLINs), indoor residual spraying (IRS); and environmental management [3,4,5]. Ethiopia has set a goal to eliminate the disease by 2030 through applying these interventions [1, 6].

The main obstacles to the control and eventual elimination of malaria globally include the presence and distribution of highly diverse *P. falciparum strains* (including drug-resistant parasites) as well as infections harboring multiple *P. falciparum* strains [7, 8, 9]. Genetic diversity in the malaria parasite is known to result from the *recombination of distinct parasite clones during zygote formation within the mosquito mid gut* [10, 11]. This diversity has the potential to alter the conformation of antimalarial drug targets and render the parasites drug-resistant [12] which will hinder malaria treatment outcome [13, 14] and reduce the efficacy of a malaria vaccine [15, 16]. Highly endemic malaria settings are prone to infections containing multiple *P. falciparum* strains, primarily due to repeated exposure to mosquitoes infected with multiple parasite strains [17]. Genetically diverse parasite species can result in persistent infections, as some parasite strains may be resistant to drug (s) used for treatment and thus survive after treatment [18] as well as enhance gametocyte production due to intra-host competition [19, 20]. Treatment failures occur more often in patients infected with higher numbers (>3 vs. <3) of *P. falciparum* strains [21, 22].

Declining malaria transmission as a result of scaling-up interventions has been shown to affect the parasite population genetics pattern and population structure of *P. falciparum* [23, 24]. However, this does not occurred in all settings. [23, 25]. Hence, accurate assessment of the parasite's genetic diversity across all susceptible populations could help plan or develop new control and elimination strategies. The multiplicity of infection (MOI), which identifies the number of clones within a particular infection, can serve as a measure of the level of malaria transmission as well as identify hotspots [26, 27].Within-host parasite diversity at the population level and the dynamics of diversity are important in malaria eradication efforts [28, 29]. Malaria parasite diversity is distinct in different individuals, populations, transmission settings and seasons within endemic zones and changes with variations in parasite prevalence [29], and has been suggested to be constantly changing [30, 31, 32, 33]. Parasite populations even respond to specific interventions, such as rapid diagnostic tests, human host immune pressure and mosquito vector [34, 35, 36]. Parasite genetic diversity and multiplicity of infection studies have also been found to be important in the surveillance of strains circulating in a particular transmission area. The identification of hotspots is important in understanding the epidemiology of *P. falciparum* infections for informed interventions to be implemented [27, 37]. This study was aimed at characterizing the genetic diversity and allele frequencies of *msp-1* and *msp-2* genes of *P. falciparum* isolates from uncomplicated malaria patients in Chewaka district, Southwest Ethiopia.

Methods

Study setting

The study was conducted in Ilu Harar Health Center, Chewka district, Buno Bedele Zone, Southwest Ethiopia during September-December 2017. Chewaka district is located in Buno Bedele zone, Oromia regional state, Ethiopia about 570 kilometers southwest of Addis Ababa. It is situated in lowland areas of Dhidhesa valley, which lies below 1500m above sea level. The district has 26 administrative *kebeles* (villages). As in most other areas, malaria transmission in Chewaka follows rainy seasons, with

transmission peaking in the months between September and December and between April and May. The main malaria control strategy in the district includes Long-Lasting Insecticidal Nets (LLINs), indoor residual spraying (IRS) and malaria case management with ACTs [2].

Study population and blood sample collection

A total of 80 *P. falciparum* blood spotted samples were collected from study participants aged six months to sixty years, who visited the health center during the evaluation of a therapeutic efficacy study of arthemeter-lumefantrine from September-December, 2017 (submitted for publication at BMC malaria Journal; Manuscript Number: MALJ-D-19-00615). The study participants who were febrile, with an axillary body temperature ≥37.5°C or having history of fever within the previous 24 hours, who fulfilled WHO revised protocol for malaria drug therapeutic efficacy study[38, 39] and signed an informed consent were included in the study. Blood samples were collected on day 0 when patients were enrolled in the study. The blood was spotted onto filter paper (Whatman® 927 mm), air-dried and stored at -20°C, for further parasite genotyping.

Extraction of parasite DNA

Genomic DNA was extracted from whole blood using proteinase K-base method (GE Healthcare Illustra Blood Genomic Prep Mini Spin Kit) according to the manufacturer's instructions for qPCR species identification and parasite density determination. For nested PCR, the DNA was extracted from stored dried bloodspots collected on enrollment (Day-0) and on any day after day 3 were deemed to have recurrent parasitaemia using Pure Link™ Genomic DNA mini Kit (Invitrogen, USA) according to the manufacturer's instructions. DNA was checked for purity and quantity using Nanodrop spectrophotometer (ND 1,000), and stored at -20 °C until used for PCR amplification and detection.

Quantitative PCR (qPCR) screening for *Plasmodium falciparum*

Primer design genesig standard Kit for Plasmodium spp. (all species) genomes was analyzed for the *in vitro* quantification of all plasmodium species genomes by targeting the 18S ribosomal RNA (18S) gene according to the protocol of Primer design™ Ltd **[**40**].** *Plasmodium falciparum* genome was analyzed for the in vitro quantification of *P. falciparum* genomes by targeting the plasmepsin 4 gene according to the protocol of Primerdesign™ Ltd **[**41**].** Each reaction was performed in duplicate and the cycle threshold number (Ct) was determined as their mean. A sample was considered positive if the fluorescent signal was detected in at least one replicate; conversely, if no signal was detected within 40 cycles, a reaction was considered negative.

Genotyping of *Plasmodium falciparum* isolates

Genotyping of *P. falciparum* isolates was carried out by Nested PCR amplification of the two highly polymorphic regions of *msp-1* (block2) and *msp-2* (block3) genes as reported previously[42, 43]. Primer sequences(Additional file 3: Table 1) and cycling parameters used for amplification of the three allelic families of *msp-1*(*K1*, *Mad20* and *RO33*) and two allelic families of *msp-2* (*FC27* and *3D7*) have been reported elsewhere [44**,** 45]. Briefly, in the initial amplification, primer pairs corresponding to conserved sequences within the polymorphic regions of each gene were included in separate reactions. The product generated in the initial amplification was used as a template in five separate nested PCR reactions. In the nested reaction, separate primer pairs targeted the respective allelic types of *msp-1*(*K1*, *Mad20* and *RO33*) and *msp-2* (*IC3D7* and *FC27*), with an amplification mixture containing 250nM of each primer, 2mM of MgCl2 and 125μM of each dNTPs and 0.4 units Taq DNA polymerase (MyTaq^{TM} DNA Polymerase, Bioline). The cycling conditions in the thermocycler (TECHNE, GENIUS), for initial *msp-1* and *msp-2* PCR were as follows: 5 min at 95 °C, followed by 25 cycles for 1 min at 94 °C, 2 min at 58 °C and 2 min at 72 °C and final extension of 5 min at 72 °C. For msp-1 and msp-2 nested PCR, conditions were as follows: 5 min at 95 °C, followed by 30 cycles for 1 min at 95 °C, 2 min at 61 °C and 2 min at 72 °C and final extension of 5 min at 72 °C[44]. The allelic specific positive control 3D7 and DNA free negative controls were included in each set of reactions [44]. Fragment analysis of *msp-1* and *msp-2* amplified products were then performed through electrophoresis on 2% and 3% ethidium bromide-stained agarose gel, respectively, and the fragments were visualized under UV light. Gel photographs were re-scored by visual comparison of DNA fragments and for individual samples, alleles were identified according to band size (Additional file 2: Figure S1; Additional file 3: Figure S2). The size of the PCR products was estimated using 100bp DNA ladder marker. We assessed the frequency of the occurrence of each allele in the population. We categorized clones into molecular weight groups differing by 20bp for clear discrimination from other clones and elimination of errors that would result from estimating the molecular weight on agarose-gels.

Data analysis

Samples from participants with multi-clonal infection produced more than one amplified fragment after either the three *msp-1* or the two *msp-2* allelic family PCR reactions [46]. Samples from participants with clonal infection produced a single product after the three *msp-1* PCR reactions as well as a single product after the two *msp-2* reactions. The *msp-1* and *msp-2* allele frequencies were expressed as the proportion of samples containing an allelic family compared to the total number of samples that gene was detected in isolates. The detection of one *msp-1* and *msp-2* allele was considered as one parasite genotype. The multiplicity of infection (MOI) was defined as the minimum number of *P. falciparum* genotypes per infected subject and estimated by dividing the number of amplified PCR fragments reflecting the parasite genotypes by the number of positive samples in the same marker [45]. The size of polymorphism in each allelic family was analyzed; assuming that one band represented one amplified PCR fragment derived from a single copy of *P. falciparum msp-1*or *msp-2* genes. Alleles in each family were considered the same if fragment sizes were within 20bp interval [47]. Spearman's rank correlation coefficients were calculated to assess association between multiplicity of infection (MOI) and geometric mean parasite

density and age. The heterozygosity index (He), which represents the probability of being infected by two parasites with different alleles at a given locus, was calculated by using the Genetic Analysis in Excel toolkit (GenAlEx) [48]. Briefly, the allelic diversity (He) for each antigenic markers was calculated based on the allele frequencies, using the formula: He= [n/ (n-1)] [(1-Σpi^2)], where n is the number of isolates sampled and pi is the allele frequency at a given locus. Allelic diversity has a potential range from 0 (no allele diversity) to 1 (all sampled alleles are different) [49]. All statistical analyses were performed using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). Differences were considered significant if *p*-value < 0.05.

Results

Demographic and parasitological data

Of the 80 patients enrolled 57 (71.2%) were males, mean (±SD) age of participants was 20.96 (±13.6) years. Participants had asexual parasitaemia ranging from 3,699 to 14, 744 parasites/μL with a geometric mean of 12,513.1parasites/μl (95% CI 12,166.9-12,859.4). The parasite DNA from the 80 *P. falciparum* samples was analyzed for *msp-1*and *msp-2* genes. The estimated frequency of *msp-1*and *msp-2* gene amplification reactions with family-specific primers was 90% (72/80) and 86.3% (69/80), respectively.

Allelic diversity of *P. falciparum msp-1* and *msp-2 genes*

Polymorphism analysis was assessed in 80 *P. falciparum* isolates within the allelic families of *msp-1* and *msp-2* with a total of 253 distinct fragments detected. The *msp-1* gene analysis showed 63, 50, 31 fragments belonged to *K1* (43.75% of overall detected *msp-1* alleles), *Mad20* (34.72%) and *R033* (21.5%) allelic families noted, respectively. The *msp-2* gene analysis showed 58, 51 fragments belonged to *FC27* (53.2% of overall detected *msp-2* alleles) and *IC/3D7* (46.8%) allelic families noted respectively.

The proportion of *K1, Mad20* and *RO33* types were 20.8, 4.2 and 4.2%, respectively. The remaining 70.8% (51/72) were polyclonal infections. Among polyclonal infections carrying two allelic types, the frequency of samples with *K1/Mad20, K1/RO33*, and *Mad20/RO33* was 31.9%, 5.6% and 5.6%, respectively. Infections with all three allelic types were detected in 29.2% of cases (Table 1)

Table 1 Genotyping of *P. falciparum msp-1* polymorphic region block 2 in malaria patients from Chewaka district, Ethiopia

msp-1, N=72	Frequency (%)	Allele size (bp)	No of alleles	Overall MOI
K1	15(20.8)	130-300	6	2.0
MAD20	3(4.2)	180-220	3	
RO33	3(4.2)	150	1	
K1+MAD20	23(31.9)			
K1+RO33	4(5.6)			
MAD20+RO33	3(4.2)			
K1+MAD20+RO33	21(29.2)			

Key: MOI; multiplicity of infection

Allele genotyping demonstrated the highly polymorphic nature (i.e. more alleles) of *P. falciparum* in Chewaka isolates with respect to *msp-1* and *msp-2(Additional file 1: Figure S1 and Additional file 2: Figure S2)*. A total of 29 individual with *msp* alleles were identified (10 for *msp-1* and 19 for *msp-2*). Among *msp-1* isolates, six *K1* (130-300 bp), three *MAD20* (180-220bp) and one *RO33* (150bp) allelic families were noted.

In *msp-2*, a total of 19 different alleles were identified (Table 2), of which ten alleles belonged to *FC27* and nine alleles belonged to *IC/ 3D7*. Allele sizes ranged from (260 to 540 bp) for *FC27* and (170 to 450 bp) for *IC/3D7* allelic families. The frequency of samples with only *FC27* and *IC/3D7* were 26.1 %(18/69) and 15.9 %(11/69), respectively. Forty of the isolates (58%) carried both *msp-2* allelic families. On the other hand, among cases that were positive for *msp-2* alleles, 42% (29/69) were monoclonal infection while 58% (40/69) were polyclonal infections.

Genotype multiplicity of *P. falciparum* infection

Of the 80 positive samples, 64 (80%) harbored more than one parasite genotype identified by the presence of two or more alleles of one or both genes. with the overall mean MOI i.e., parasite clones per sample was 3.2 (95% CI: 2.87- 3.46). When considering *msp-1* and *msp-2* genes separately, the MOI was 2.0 (95% CI: 1.82-2.18) and 1.6 (95% CI: 1.46- 1.70), respectively, while 51/72(70.9%) and 40/66(58%) of isolates contained multi-clonal infection at least with 2 clones, respectively. The heterozygosity index, which represents the probability of being infected by two parasites with different alleles at a given locus, was 0.43 for *msp-1*and 0.85 for *msp-2* loci. No significant correlation between multiplicity of infection and parasite density of patients (Spearman rank correlation = 0.094; p= 0.409) or multiplicity of infection and age (Spearman rank correlation = 0.072; p= 0.528).

Table 2 Genotyping of *P. falciparum msp-2* polymorphic region block 3 in malaria patients from Chewaka district, Ethiopia

msp-2,N=69	Frequency (%)	Allele size(bp)	No of alleles	Overall MOI
FC27	18(26.1)	260-540	10	1.6
IC/3D7	11(15.9)	170-450	9	
FC27+IC/3D7	40(58.0)			

Key: MOI, multiplicity of infection

Discussion

The *P.falciparum* malaria risk index in Chewaka district is classified as moderate transmission with an annual parasite incidence (API) of >=5 to <100 case per 1000 population [3]. The declining malaria transmission, as a result of scaling up interventions, has been shown to affect the genetic diversity pattern and population structure of *P. falciparum* [23, 24].As Ethiopia moves towards malaria elimination, understanding the genetic diversity and population structure of the malaria parasite populations in hotspots is crucial to guide monitoring and evaluation of malaria control strategies and antimalarial interventions. The present study provides detailed assessment of genetic diversity and multiplicity of infection of *P. falciparum* parasites from Chewaka district, southwest Ethiopia.

Allelic specific PCR typing of *msp-1* and *msp-2* genes showed a considerable allelic diversity in the *P. falciparum* population in the analyzed samples. Indeed, the numbers of alleles (bands) detected may be underestimated due to sensitivity of the PCR technique used as minor fragments (<50 bp) cannot be detected on the agarose gel. The number of alleles detected for *msp-1* and *msp-2* were 10 and 19, respectively *(Additional file 1: Figure S1 and Additional file 2: Figure S2)*, which is high compared with the reported values in other areas of Ethiopia [46, 50].

In this study, allelic specific PCR typing of *msp-1* gene showed the presence of *K1*, *Mad20* and *R033* allelic families in the 80 positive samples amplified. The *K1* family was predominant (43.75%), followed by Mad20 (34.72%) and RO33 (21.5%). We found that of the 72 allelic types detected in *msp-1,* the K1 allelic family with 20.8% allelic frequency besides its representation in poly allelic bands was predominant. This is in line with a previous study from Kolla-Shele area, Arbaminch Zuria District, southwest Ethiopia [46] and studies in Ghana and Benin [51, 52]. However, studies in Northern Ethiopia [50] and Malaysia [53] reported *Mad20* allele was predominant. Variations in the prevalence of block 2 alleles between different studies likely reflects the differences in geographic locations and local transmission intensity [54, 55]. The *RO33* family was poorly polymorphic with only one allele (160bp). This is in line with studies from Congo [56], but in contrast to the previous findings in other regions of Ethiopia [46, 57].

At the locus *msp-2*, 69 allelic types were found (18 *FC27*, 11 *IC/3D7* and *40 FC+IC/3D7*). *FC27* and *IC/3D7* was the most polymorphic with 19 different alleles. However, the *FC27* family was more frequently detected 18(26.1%) in our study. This is consistent with reports from studies in Benin [51] and Sudan [58] but differs from previous study in Ethiopia [57] and Brazzaville, Republic of Congo [47]. This suggests that the diversity of *P. falciparum* infections may differ according to geographic location, transmission intensity and sample population.

Our results indicate that *msp-2* was more polymorphic than *msp-1.* This genetic marker is the best for estimating the multiplicity of *P. falciparum* infection in symptomatic patients because multiplicity of infection is significantly related to parasite density [59]. However, in this study we did not find significant relation-ship between an allelic distribution and the parasite count *($p < 0.05$).* Furthermore, genetic diversity values were higher based on heterozygosity index for *msp-2* (He=0.85), than for *msp-1* (He=0.43), suggesting a large genotype diversity within the *msp-2* locus, which was higher than previously reported in Ethiopia [46]. In contrast, declining rates of diversity of alleles (heterozygosity) in *P.falciparum* area associated with decreasing transmission rates [54**,** 60]. Transmission is another critical step for the spread of drug-resistant parasites. Drug-resistant parasites emerging in a high-transmission area would likely be present in a polyclonal infection i.e. up to 7 clones have been reported to coexist within one host [61]. Therefore, this finding suggests a relatively high diversity of the *P. falciparum* parasite population in Chewaka district, Ethiopia suggesting that to pre-empt the emergence or spread of resistance to anti-malarial drugs, the genotype and phenotype of parasites from all malaria endemic populations need to be regularly monitored.

In this study, we have reported that 80% of the isolates harbored more than one parasite genotype identified by the presence of two or more alleles of one or both genes with the overall mean MOI i.e., parasite clones per sample, being 3.2 (95% CI: 2.87- 3.46). When considering *msp-1* and *msp-2* genes separately, the mean MOI was 2.0 and 1.58 respectively, while 51/72(70.8%) and 40/69(58%) of isolates contained multi-clonal infection at least with 2 clones, respectively. This was higher compared to those reported from a moderate transmission settings in Kolla-Shele, Southwest Ethiopia [46], but consistent with findings from Northwest Ethiopia [50]. Despite the lack of entomological data such as entomological inoculation rate (EIR) from Chewaka district, the number of clones co-infecting a single host can be used as an indicator of the level of malaria transmission or the level of host acquired immunity [54, 59, 60]. Besides, transmission intensity can also be affected by other factors such as vector biting behavior and endemicity [54]. Inferring high transmission intensity from the presence of multi-clonal infections alone has additional limitations including estimates of MOI varying by genotyping method, potential impact from sampling frequency and a non-linear relationship between MOI and transmission intensity [54]. Despite these limitations, infection with multiple clones observed in this study, combined with evidence of high genetic diversity would be consistent with high transmission intensity in the study area. This finding is consistent with an association between malaria transmission levels and the frequency of multiple infections, with individuals living in high malaria transmission areas generally harboring multiple parasite strains and thus it requires increased attention by malaria control programs.

Strengths And Limitations Of The Study

The present study has vigorously genotyped the *P. falciparum* isolates on 2 antigenic (*msp-1* and *msp-2*) markers with efforts to obtain a conclusive genetic diversity dataset for the areas. The limitation of the present study is the small sample size collected by active case detection during the evaluation of a therapeutic efficacy study of arthemeter-lumefantrine in the area. Despite this limitation, the data from the present study has confirmed the high genetic diversity profile and mixed-strain infections of *P. falciparum* populations in Chewaka district, Ethiopia; reflecting both the endemicity level and malaria transmission remained high, key malaria hotspot area in southwest, Ethiopia.

Conclusions

The study presented baseline genetic diversity data of *P.falciparum* isolates in malaria hotspot area of Chewaka district, Ethiopia prior to the targeted elimination of malaria by 2030. The high level of polyclonal infections with *P. falciparum* parasites harbored multiple genotypes and also infections of higher MOIs in this study indicate the extensive genetic diversity and *P. falciparum* infection complexity in Chewaka district, Southwest Ethiopia. More effort is needed to control malaria transmission and prevent the emergence of resistance alleles in the study area.

Declarations

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Authors' contributions

AbAb, DaYi, DeYe, AGB, HN, AA conceived and designed the study. AbAb, HE, WA, MM performed the experiments. AbAb analyzed the data and drafted the manuscript. AbAb, HE, WA, AI, DaYi, AGB, DeYe, HN, MM, AA reviewed the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

The data sets generated and /or analyzed during the current study are included within the article and its additional files.

Ethical considerations

The study protocol was approved by the Ethics Review Board of Jimma University. Written informed consent from adult participants and parents/guardians of the study children and assent from children aged less than 18 years was obtained for all participants. If a patient, parent or guardian was illiterate, impartial witness was used.

Competing interests

The authors declare that they have no competing interests.

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Consent for publication

All authors have given their consent for publication

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