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Plasmodium falciparum merozoite surface protein 2 genetic polymorphism and multiplicity of infection in selected malarious areas of Northwest Ethiopia

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Abstract

Background The genetic diversity of *Plasmodium falciparum* provides the parasite with many effective immune evasion and drug resistance mechanisms. This phenomenon is a major problem in eradicating malaria globally. This study aimed to assess merozoite surface protein 2 polymorphisms in *P. falciparum* isolates from Northwest Ethiopia.

Method A cross-sectional study was conducted to assess merozoite surface protein 2 polymorphisms in *P. falciparum* isolates from selected malarious areas in Northwest Ethiopia from April to June 2021. A convenience sampling technique was used to select 150 study participants. A finger prick blood sample was collected to prepare blood films and dried blood spots for molecular genotyping. The merozoite surface protein 2 allele frequency and multiplicity of infection were computed. Linear regression was employed to evaluate the associations between the multiplicity of infection, parasite density, and age by calculating Spearman's rank correlation coefficients. A P value < 0.05 was considered to indicate statistical significance.

Result Polymorphism analysis was performed on 126 *P. falciparum* isolates. There were 38 different merozoite surface protein 2 alleles, 20 of which corresponded to the IC/3D7 allelic family and 18 to the FC27 allelic family. Most patients contained multiple infections, and the mean multiplicity of infection was 3.46. There was no statistically significant difference in the multiplicity of infection in relation to the age of patients ($P = 0.646$). However, a statistically significant correlation was found between parasite density and the multiplicity of infection ($P = 0.046$). The heterozygosity index for merozoite surface protein 2 was 0.948.

Conclusion This study showed that *P. falciparum* isolates contain multiple genotypes with a high multiplicity of infections and mixed strain infection, suggesting extensive genetic diversity and a high level of malaria transmission. This genetic variability could complicate malaria treatment and control efforts, as it can facilitate the emergence and spread of drug-resistant strains. Consequently, the findings highlight the complex malaria epidemiology in the region

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and emphasize the need for intensified efforts to control malaria transmission and prevent the probable emergence of drug resistance alleles in the study area.

Keywords Merozoite surface protein 2, Polymorphism, Genetic diversity, *Plasmodium falciparum*, Gondar, Northwest Ethiopia

Background

Malaria is a serious infectious parasitic disease caused by several *Plasmodium* species [1], mainly transmitted through the bites of infected female *Anopheles* mosquitoes [2]. There are five *Plasmodium* species (namely, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, *and Plasmodium knowlesi*) that cause malaria in humans [1]. Among these, *P. falciparum* and *P. vivax* are the most significant threats due to their widespread prevalence and potential for severe disease [3]. Globally, malaria remains a serious public health problem, with an estimated 249 million cases reported across 85 endemic countries [4], leading to significant morbidity and mortality [2]. The burden is particularly heavy in low-income countries, especially in Africa, where 236 million cases (95% of global cases) and 590,935 deaths (97% of global deaths) were reported [4]. In Ethiopia, malaria is a major public health concern, with 2.78 million cases and 8,041 deaths reported, despite ongoing control and preventive strategies implemented [2].

Controlling malaria is increasingly challenging due to the emergence of drug and insecticide resistance [5], driven by genetic polymorphisms that create diverse parasite populations [6]. *P. falciparum*, the most virulent malaria parasite species [7], is particularly prone to developing resistance to antimalarial drugs, posing a major challenge in the fight against malaria [8]. Its high genetic diversity complicates control efforts, enhances immune evasion, contributes to the severity of malaria, and leads to the emergence of drug-resistant variants [9]. This diversity arises from mechanisms, such as allelic polymorphisms, chromosome rearrangements, genetic recombination within the mosquito during the parasite's lifecycle, and antigenic variation [10].

Genotyping is essential for understanding the genetic structure of parasite populations and developing effective malaria control measures [11]. *P. falciparum* exhibits significant genetic variability. Amplifying and analyzing the merozoite surface protein 2 (MSP2) gene on chromosome 2 of *P. falciparum* is commonly used to study its genetic variability. This gene has five blocks, with block 3 being the most polymorphic [12], making it effective for assessing parasite population diversity [13]. MSP2 gene alleles are categorized into two major families: FC27 and IC/3D7 [14]. Genotyping using the MSP2 gene reveals allelic variability and determines the multiplicity of infection (MOI), indicating the number of clones per isolate [15]. *P. falciparum* genetic structures and population genetic studies may also play a key role in successful disease surveillance and control programs [16], particularly in northwestern Ethiopia, where there is limited information available. This study aims to investigate the MSP2 genetic polymorphisms of *P. falciparum* in Northwest Ethiopia to fill the knowledge gap and contribute to understanding the genetic diversity of malaria parasites in this area, which is essential for developing targeted interventions and improving malaria management strategies.

Methods and materials

Study area

A cross-sectional study was conducted among malaria patients from Northwest Ethiopia at the Koladiba, Sanja, and Zarima Health Institutions (Fig. 1) from April 10, 2021, to June 25, 2021. The selected areas are known for high malaria transmission [17–19]. The Koladiba district is located 35 km from the town of Gondar in the Amhara Region of Ethiopia. The total population is estimated to be 307,967 in an area of 148,968 sq. km. Its altitude ranges from 1,850 to 2,000 m a.s.l, with an annual rainfall of 700 to 1,160 mm. The annual temperature ranges from 18 to 30 °C. The district has one hospital, 10 health centers, 40 health posts, and 20 private clinics at its services [20].Among the ten health centers, Koladiba Primary Hospital was chosen for this study. On the other hand, Sanja Hospital, which is located in Sanja town, central Gondar Zone, was the second study area. Sanja is the capital of the Tach Armachiho district, which is surrounded by the Maho Stream and Sanja River. The town is located 65 km from Gondar town and 792 km from Addis Ababa in the northwestern part of Ethiopia. Sanja is located at an altitude of 1800 m above sea level, with annual rainfall ranging from 800 to 1800 mm and temperatures ranging from 25 °C to 42 °C [21]. One health center and one hospital provide services for the residents of the town and surrounding areas. Zarima is one of the small towns in Adarkay Woreda in North Gondar, which is 140 km away from Gondar town. The town has a population of approximately 20,000 people. The altitude of the town ranges from 1000 to 2400 m a.s.l [22]. Health facilities were selected based on records of higher malaria cases at their respective locations.

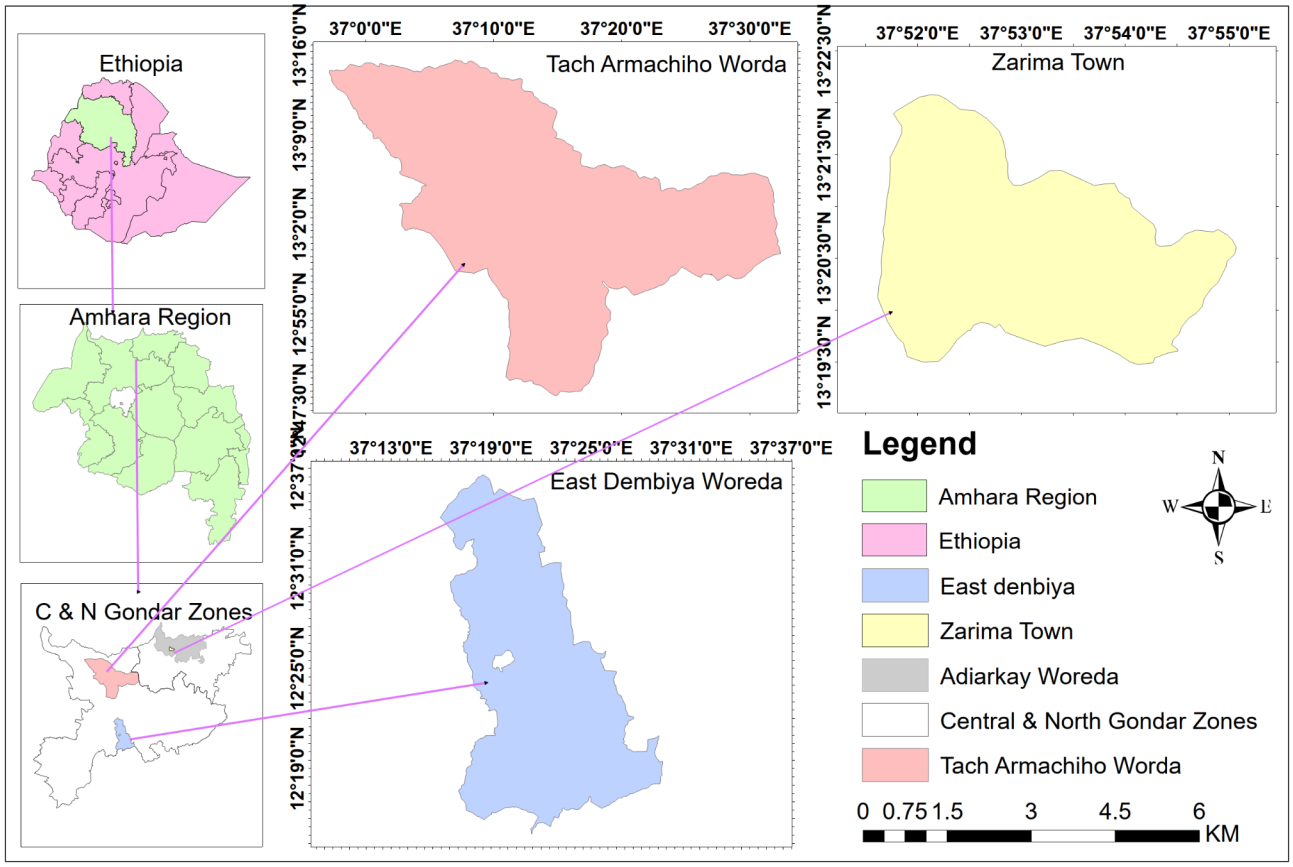

Fig. 1 Map of the sample collection area, Koladiba/Koladiba, Sanja, and Zarima, Northwest, Ethiopia

Study population and blood sample collection

Finger prick blood samples were collected aseptically from the fingers of 520 febrile patients. Before blood sample collection, the finger was cleaned with alcohol-moistened cotton. Thick and thin blood smears were prepared on slides and air-dried, and the thin films were fixed with methanol. The slides were labeled, and stained with 10% Giemsa solution for 10 min. After staining, the slides were washed with distilled water and left to air-dry once more. Finally, the slides were examined by laboratory technicians to identify *Plasmodium* species. *P. falciparum*-positive slides were transported to the medical parasitology laboratory at the University of Gondar for parasite load quantification by counting asexual parasites per 200 white blood cells (WBCs), with an assumed white blood cells level of 8,000/µl [23]. Additionally, 50 µl blood samples from 150 confirmed *P. falciparum* patients were spotted on Whatman™ 903 filter paper [24], directly from the pricked finger, air-dried at room temperature, and placed in plastic bags containing silica gel desiccants. Molecular identification of the *Plasmodium* spp. and MSP2 genotyping were carried out at the Armauer Hansen Research Institute (AHRI) laboratory, Addis Ababa, Ethiopia.

Nucleic acid purification and molecular analysis

Genomic DNA was extracted from dried blood spots by using the combined 0.5% Tween-20/Chelex-100 technique [25]. *Plasmodium* spp. were identified by 18 S rRNA gene-based nested PCR using genus-and species-specific primers as described by Snounou et al. 1993 [26].

The MSP2 allelic families were determined using nested PCR described elsewhere [27]. PCR confirmed *P. falciparum* samples were selected for MSP2 genotyping in parallel with the known FC27 and 3D7 allelic family positive controls and negative control (molecular grade water) in each experiment.

Each *P. falciparum* infection was characterized based on the fragment size of the PCR products for MSP2. Infections are defined as polyclonal if parasites from a single patient show more than one allele of a gene. If an isolate has one allele, the clone number is taken to be monoclonal. The MOI was calculated as the average number of distinct fragments per PCR-positive sample [28]. The frequency of each allelic type was estimated using the presence of PCR products for each allelic type in the total number of amplified bands for the corresponding locus. Individual alleles were differentiated based on fragment length and the use of allele-specific primers. A 100 bp DNA ladder marker was used

Table 1 Demographic and parasitological data of the study individuals at Koladiba, Sanja, and Zarima, North West Ethiopia, 2021

Characterization of patients	Values
Mean age (year)	median (20), interquartile range (14, 30) years
Study participant's ages ranged	2 to 80 years
Sex ratio (Male/Female)	1.25(70/56)
Geometric mean of parasite density (p/μl)	5417 Parasites/μl (95% CI 4411–6815)
Parasite density range (p/μl)	480–48,120 Parasites/μl

p/μl: parasite per microliter

to determine the size of the PCR products [29]. Alleles in each family were considered identical if the fragment sizes were within a 20 bp range [30]. Agarose gel images were rescored via visual comparison of DNA fragments, and alleles were identified for individual samples based on band size. The size polymorphism in each allelic family was examined under the assumption that one band represented one amplified PCR fragment generated from a single copy of the *P. falciparum* MSP2 gene.

Data analysis

Data were entered into Microsoft Excel worksheet 20, cleaned, and exported to SPSS V 20 (IBM Corp. IBM SPSS Statistics for Windows, Armonk, NY, USA). The frequency of MSP2 alleles was determined as the proportion of alleles in the allelic family. Linear regression assessed relationships between multiplicity of infection, parasite density, and age, using Spearman's rank correlation coefficients. Heterozygosity (He) was determined using genetic analysis in Excel tools (GenAlEx), which was computed by using the following formula: He = [n/(n-1)] [(1-Σpi2)], where n is the number of alleles in the sample and pi is the frequency of each distinct allele at each locus [31]. *P* values < 0.05 indicated statistical significance.

Results

Parasitological and demographic data

In this study, 520 febrile individuals with different age groups of both sexes were screened until 150 *P. falciparum* microscopically confirmed malaria cases were obtained (Table 1). Among 150 microscopically confirmed *P. falciparum*-positive samples, 132 gave a signal for *P. falciparum* confirmed by nested PCR. The MSP2 gene was successfully amplified in 126 samples, but six samples were excluded due to faint band intensity during nested PCR species identification, even after retesting.

Table 2 *P. falciparum* MSP2 allele genotype and multiplicity of infection among malaria patients from Koladiba, Sanja, and Zarima, Northwest Ethiopia, 2021

MSP2, *N* = 126	Frequency (%)	Allele size(bp)	Number of alleles	Overall MOI
FC27	0	265–665	18	3.46
IC/3D7	3(2.4)	260–720	20	
FC27 + IC/3D7	123(97.6)	265–665/260–720		
Total	126		38	

N = number, MOI = multiplicity of infection

Table 3 The expected heterozygosity for *P. falciparum* MSP2 alleles in malaria patients from Koladiba, Sanja, and Zarima, Northwest Ethiopia, 2021

Expected heterozygosity for IC/3D7 (HE)	Expected heterozygosity for FC27 (HE)	Mean Expected heterozygosity (HE)
0.957	0.939	0.948

Genetic diversity and allelic frequency of the *P. falciparum* MSP2 gene

Overall polymorphism analysis was performed on 126 *P. falciparum* isolates within the MSP2 allelic families, yielding 436 unique fragments. Among those fragments, 250 (57.4%) and 186 (42.6%) fragments belonged to the IC/3D7 and FC27 allelic families, respectively. Allelic genotyping of the MSP2 gene of *P. falciparum* in Northwest Ethiopia revealed its polymorphic nature. The frequencies of IC/3D7 and FC27 were 100% (126/126) and 97.6% (123/126), respectively. There were thirty-eight different MSP2 alleles, 20 of which corresponded to the IC/3D7 allelic family and 18 to the FC27 allelic family. Allele sizes ranged from 265 to 665 bp for FC27 and 260–720 bp for the IC/3D7 allelic families. The proportion of isolates with only IC/3D7 alleles was 3 (2.4%), and no isolates had only FC27 alleles. One hundred twenty-three (97.6%) of the isolates carried both allelic families (Table 2). Three isolates were not identified as members of the FC27 allelic family despite being repeatedly amplified. The heterozygosity (He) index determined for the antigenic markers of MSP2 was 0.948 (Table 3). For individual sites, polymorphism analysis of the MSP2 gene in *P. falciparum* isolates from Koladiba, Sanja, and Zarima revealed the presence of both IC/3D7 and FC27 allelic families across all sites. Koladiba had 39 isolates with 143 unique fragments (79: IC/3D7 and 64: FC27), Sanja had 47 isolates yielding 169 fragments (93: IC/3D7 and 76: FC27), and Zarima had 40 isolates with 124 fragments (78: IC/3D7 and 46: FC27). The IC/3D7 allelic family was more prevalent, comprising 55.3%, 55.1%, and 62.9% of the unique fragments in Koladiba, Sanja, and Zarima, respectively. All isolates carried both allelic families, with

24 (12: IC/3D7 and 12: FC27 family), 23(12: IC/3D7 and 11: FC27 family), and 25 (10: IC/3D7 and 15: FC27 family) different MSP2 alleles identified in the respective sites. In Zarima, all isolates had IC/3D7 alleles, but 92.5% had FC27 alleles, indicating that 3 isolates (7.5%) carried only IC/3D7 alleles.

Multiplicity of *P. falciparum* infection

Among the 126 positive samples, 123 (97.6%) isolates carried both the IC/3D7 and FC27 alleles, with an overall MOI of 3.46 (95% CI 3.35–3.56). For individual sites, the MOI was 3.58 (95% CI 3.25–3.75), 3.72 (95% CI 3.6–385), and 3.10 (95% CI 2.97–3.28) in Koladiba, Sanja, and Zarima, respectively. These findings suggest a high prevalence of MOI, with an average of 3–4 distinct parasite genotypes per infected individual. There was no statistically significant difference in the number of infections by age (Spearman rank correlation – 0.04, $p = 0.646$). However, a statistically significant correlation was found between parasite density (Spearman rank correlation 0.178, $p = 0.046$) and the number of genotypes. The parasite density and multiplicity of infection decreases as the age of the patient increases (Table 4).

Table 4 Mean *P. falciparum* density (parasite/μl) and multiplicity of infection in the MSP2 gene stratified by age group ($N = 126$) in malaria patients from Koladiba, Sanja, and Zarima, Northwest Ethiopia, 2021

Age (years)	*N*	The geometric mean of Parasite density/μL	MOI
< 5	8	12,436	3.50
5–15	29	5558	3.52
> 15	89	4985	3.44

N: number of malaria cases, MOI: multiplicity of infection

Discussion

Malaria transmission and management strategies are affected by the genetic diversity of *P. falciparum* parasites [32]. Thus understanding this diversity across different geographical settings is crucial for determining the parasite's population structure and designing effective control strategies [33]. Although significant efforts have been made to control and eventually eliminate malaria in Ethiopia still the disease continues its transmission. Hence, supporting epidemiological studies with molecular data on *P. falciparum* genetic polymorphisms, is important to aid malaria control. Thus, this study assessed polymorphisms of the MSP2 gene in samples from different study sites in Northwest Ethiopia to generate scientific evidence.

The study found that the predominant alleles for the MSP2 gene in the area were 3D7/IC. This finding is consistent with reports from South Benin [34], Bobo-Dioulasso, Burkina Faso [28], and Adama, Ethiopia [35]. In contrast, the FC27 family is the most frequent genotype in the Chewaka district, Ethiopia [16], Kosti, central Sudan [36], and Khyber Pakhtunkhwa, Pakistan [37]. The difference in allelic distribution could vary based on geographical location, transmission intensity, and the size of the study population, which influence the local adaptation of allelic families [13]. Transmission intensity significantly influences the allelic distribution, with high transmission areas exhibiting greater genetic diversity due to multiple co-circulating parasite strains and polyclonal infections, allowing advantageous alleles to thrive. Conversely, low transmission results in reduced diversity and monoclonal infections dominated by specific alleles. The dominance of IC/3D7 alleles is likely due to genetic recombination, which increases allele frequency and biological fitness [38]. Furthermore, balancing selection may play a role [12] in maintaining multiple alleles at a locus due to their advantages under different host immune pressures. The size of the study population has a significant impact on the allelic distribution, with larger populations capturing more genetic diversity and improving allele frequency estimates, while smaller populations may fail to detect rare alleles and are more prone to sampling bias [13] Additionally, geographic variations in allele frequencies further reflect local immune adaptations [39], influencing the prevalence of specific MSP2 alleles in different regions.

Allele-specific genotyping of MSP2 showed high genetic diversity in the *P. falciparum* population in this study area, which may indicate ongoing transmission despite the intensification of malaria control measures [40]. This finding was higher than the study found in Humera, Northwest Ethiopia [41], Melka-Werer, Northeast Ethiopia [42], Kosti, Central Sudan [36], but lower than China [43] and North Central Nigeria [44]. The genetic diversity could vary depending on geographical location, transmission intensity, malaria treatment policies, and sample population [13, 45]. The high genetic diversity in parasites from high transmission areas may be due to genetic recombination [46], which generates novel beneficial allele combinations that can spread through the population under positive selection [12]. More malaria-endemic sites had greater genetic diversity than lower malaria-endemic sites [47], indicating that the level of endemicity determines *P. falciparum's* genetic diversity. Moreover, treatment policies that focus on the use of highly potent drugs targeting both asexual blood-stage parasites and gametocytes are more effective than drugs acting only on blood-stage parasites. Highly potent drugs effectively decrease parasite transmission and clonal diversity [48], which may impact the parasite's genetic variability, adaptive ability, and opportunities for sexual recombination [49].

Heterozygosity (HE) and multiplicity of infection (MOI) are indirect measures of parasite genetic diversity.

The heterozygosity observed in this study was notably high (He = 0.948), suggesting a significant genotype diversity within the MSP2 locus, higher than previously reported in Ethiopia [13, 41, 42]. This shows a high transmission pattern of distinct *P. falciparum* clones. Genetically distinct clones may adapt better to existing interventions and increase the likelihood of developing antimalarial resistance [50]. Furthermore, the present study demonstrated a significant level of multiclonal *P. falciparum* infection, with 96.7% of isolates containing multiple genotypes, indicating high diversity and transmission in the area. This suggests that the scaling up of malaria control efforts may not have effectively reduced the levels of multiplicity of infection, which could be due to multiple mosquito bites transmitting different genotypes or the inoculation of genetically diverse sporozoites from a single mosquito bite [12].

The overall MOI in *P. falciparum*-infected malaria patients was 3.46. This was higher than studies conducted in China [43], Bobo-Dioulasso, Burkina Faso [28], North Central Nigeria [44], Adama, Ethiopia [35], and Humera, Northwest Ethiopia [41], but lower than that reported in West Benegal, India [51] and South Benin [34]. The variation could be due to the level of malaria endemicity, transmission intensity, and other factors, such as the age of the study population, parasite density in the study population, use of different antimalarial drugs, vector populations, and immune status [32, 37, 52, 53]. High-transmission areas have a greater multiplicity of infections, while low-transmission regions have more single-clone infections. A high MOI indicates polyclonal infections and intense malaria transmission, driven by larger vector populations facilitating superinfections or simultaneous transmission of different parasite genotypes [54]. Vector populations have a significant impact on the MOI in *P. falciparum* by feeding on multiple hosts, which increases the likelihood of exposure to different strains of the parasite and thus raises the MOI [53]. Despite antimalarial therapies are expected to reduce various parasite strains' diversity [55], this study revealed high levels of MOI suggesting any possible contribution of drugs to reduce diversity may have been insignificant [45]. The patient's immune status is also key in determining MOI; high levels of multiple clones reflect a low level of acquired immunity and a poor infection control capacity. Stronger immune responses in individuals with high exposure can limit the number of strains, while weaker responses in vulnerable populations lead to greater strain diversity and persistence [34].

The relationship between age and MOI in *P.falciparum* malaria patients is complex, with some studies showing a negative correlation due to older individuals having more anti-parasitic immunity [56] while others report a positive correlation in younger populations with lower immunity [32]. Our research found no significant influence of age on MOI, consistent with findings from other studies [14, 44]. This suggests that MOI is not directly related to the duration of immune acquisition in asymptomatic patients but reflects the exposure of subjects to malaria in the endemic area [37]. Other factors, such as parasite density significantly impact MOI, as higher densities increase the likelihood of diverse parasite genotypes. This relationship is influenced by host immunity, the number of available clones, and other ecological and biological factors [47]. Our findings indicate a positive correlation between MOI and parasite density, consistent with reports from Khyber Pakhtunkhwa, Pakistan [37], Bioko Island, Equatorial Guinea [32], Kosti, Central Sudan [36], Adama, Ethiopia [35]. However, some studies show a negative correlation, due to competition among genetically distinct strains within the host, which can suppress the presence of co-infecting variants [42] A better understanding of this relationship can enhance malaria control efforts and provide insight into the transmission and evolution of the parasite. However, the study has limitations, including reliance on a single blood sample, which may not be enough to show the whole diversity of parasites carried by an individual since genotypes can appear and disappear quickly. Additionally, using a single antigenic marker may underestimate the diversity of infections. Despite these limitations, the study provides valuable insights into the genetic diversity of *P. falciparum* in Northwest Ethiopia, which is crucial for informing policymakers in developing effective, region-specific malaria prevention and control strategies. Additionally, it provides essential baseline data for future epidemiological research on malaria transmission at the site.

Conclusion

The present study showed that *P. falciparum* isolates contain multiple genotypes with high heterozygosity. Among the polymorphic alleles IC/3D7 has a high frequency indicating its better adaptation in the study area. It is also indicated that there is a high level of multiplicity of infection influenced by parasite density but not influenced by age variation. Parasite load is associated with high genetic polymorphism. The prevalence of multiclonal infections indicates a complex parasite population and high malaria transmission rates. This genetic diversity poses challenges for malaria treatment and control, as it can facilitate the emergence and spread of drug-resistant strains. Overall, the findings highlight the complex malaria epidemiology in the region and emphasize the need for intensified efforts to control malaria transmission and prevent the emergence of resistance alleles in the study area. To achieve this goal, the governmental and non-governmental stakeholders in the areas must

work together more to achieve the intended malaria control and prevention efforts. Specifically, the district and zonal health offices should focus on increasing public awareness regarding malaria prevention practices, such as the correct use of bed nets and the importance of early diagnosis and treatment. Additionally, the regional health bureau should prioritize and allocate more resources to this area for malaria control measures, including mass drug administration, indoor residual spraying, and the distribution of insecticide-treated bed nets. Furthermore, researchers should conduct investigations using large sample sizes and advanced techniques such as microsatellite DNA sequencing and capillary electrophoresis, focusing on markers such as MSP2, as well as other markers such as MSP1 and glutamate-rich protein (GLURP), to draw comprehensive conclusions.

Abbreviations

AHRI	Armauer Hansen Research Institute
a.s.l.	Above sea level
Bp	Base pair
DNA	Deoxyribonucleic acid
HE	Mean Expected heterozygosity
MSP	Merozoite Surface Protein
MOI	Multiplicity of Infection
PCR	Polymerase Chain Reaction
rRNA	Ribosomal Ribonucleic Acid

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12879-025-10676-1.

Supplementary Material 1

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Author contributions

AA, MA, AA, MG, JD, DAM, TE, MK and MA were involved in the study conception, data analysis, and drafting of the manuscript. AA and AA collected and processed the samples. AA, AA, TE, MK and MA reviewed the manuscript. All authors have read, edited, and approved the manuscript.

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Data availability

All the data is included in the manuscript.

Declarations

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Ethics approval

Ethical approval and a permission letter were obtained from the ethical review committee of the School of Biomedical and Laboratory Science at the University of Gondar on April 7, 2021, with the reference number SBMLS/2791. Moreover, a permission letter for conducting the study was also obtained from the respective health institution directors. The Helsinki ethical code principle was followed and hence informed verbal consent and assent were obtained from the study participants and the children's parents/guardians, respectively. Study subjects who were found malaria-positive had been treated according to the country's drug regimen policy.

Conflict of interest

The authors declare no competing interests.

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