Malaria Journal

RESEARCH

Open Access

Novel *Plasmodium falciparum* histidine-rich protein 2/3 repeat type in Ethiopian malaria infection: does this affect performance of HRP2-based malaria RDT?

Aynalem Mandefro[1,2*], Alebachew Messele Kebede[1], Bacha Mekonen[3], Mitchel Katsvanga[4], Fatoumatta Cham[4], Blessed Etoketim[4], Eniyou Oriero[4], Alfred Amambua-Ngwa[4] and Lemu Golassa[1*]

Abstract

Background Rapid diagnostic tests (RDTs) provide quick, easy, and convenient early diagnosis of malaria ensuring better case management particularly in resource-constrained settings. Nevertheless, the efficiency of HRP2-based RDT can be compromised by *Plasmodium falciparum* histidine-rich protein 2/3 gene deletion and genetic diversity. This study explored the genetic diversity of PfHRP2/3 in uncomplicated malaria cases from Ethiopia.

Methods A cross-sectional study was conducted from June 2022 to March 2023 at Metehara, Zenzelema and Kolla Shele health centres, Ethiopia. Finger-prick blood samples were collected for RDT testing and microscopic examination. For molecular analysis, parasite genomic DNA was extracted from venous blood. *Plasmodium falciparum* was confirmed using *VarATS* real time PCR. Additionally, PfHRP2/3 was amplified, and DNA amplicons were sequenced using Oxford Nanopore technology.

Results PfHRP2/3 sequences revealed small variations in the frequency and number of amino acid repeat types per isolate across the three health centres. Twelve and eight types of amino acid repeats were identified for PfHRP2 and PfHRP3, respectively, which had been previously characterized. Repeat type 1, 4 and 7 were present in both PfHRP2 and PfHRP3 amino acid sequences. Type 2 and 7 repeats were commonly dispersed in PfHRP2, while repeat types 16 and 17 were found only in PfHRP3. A novel 17 V repeat type variant, which has never been reported in Ethiopia, was identified in six PfHRP3 amino acid sequences. The majority of the isolates, as determined by the Baker's logistic regression model, belonged to group C, of which 86% of them were sensitive to PfHRP2-based RDT. Likewise, PfHRP2-based RDT detected 100% of the isolates in group A (product of type 2 × type 7 repeats ≥ 100) and 85.7% in group B (product of types 2 × type 7 repeats 50–99) at a parasitaemia level > 250 parasite/µl.

Conclusion This study highlights the significant diversity observed in PfHRP2 and PfHRP3 among clinical isolates of *Plasmodium falciparum* in Ethiopia. This emphasizes the necessity for monitoring of PfHRP2- based RDT efficacy and their repeat type distribution using a large sample size and isolates from various ecological settings.

Keywords Malaria, RDT, PfHRP2/3, Amino acid sequence, Repeat type, Ethiopia

*Correspondence:
Aynalem Mandefro
anaafman@gmail.com
Lemu Golassa
lgolassa@gmail.com
Full list of author information is available at the end of the article

Background

Although there has been a declining trend in malaria prevalence in the last two decades, recent data shows an alarming increase in both malaria-related morbidity and mortality worldwide. In 2022, approximately 249 million cases and 608,000 cases were reported globally [1]. The impact of this preventable disease is particularly pronounced in Africa, where over 94% global cases and fatalities occur [1]. Climate change, the emergence of anti-malaria drug resistant parasites and insecticide-resistant vectors, the COVID-19 pandemic, and population displacement due to drought and civil stress are among the key factors fueling the recent upsurge in global malaria cases [1, 2]. Malaria is a major public health problem in Ethiopia, where around 60% of the population resides in malaria-prone areas [3, 4]. Ethiopia reported 2.4 million cases of malaria in 2022, [1], with evidence of resurgences in some parts of the country [5].

Malaria control relies on the use of test and treat strategies, with a focus on precise diagnosis before treatment recommendations are made [1, 6]. Globally, microscopic examination of blood film is considered as the gold standard diagnostic tool in numerous health settings, including Ethiopia [3, 6]. Nevertheless, its utilization in resource-limited areas is hindered by challenges such as in adequate infrastructure, shortage of qualified personnel, unstable electric supply, and lack of high-quality reagents [7, 8]. Conversely, malaria rapid diagnostic tests (RDTs) have overcome the limitations of microscopy and offer a convenient, easy, and quick way for early diagnosis in resource-constrained settings [8, 9]. RDTs have improved case management across Ethiopia since its introduction in late 2004, particularly at peripheral health settings or health posts [3], where there is no electricity and hence microscopic diagnosis is impractical. Approximately 10 million RDTs are used annually in the country, with 70% of health facilities that provide malaria diagnosis utilizing RDTs [10].

Rapid diagnostics tests utilize nitrocellulose membrane-impregnated monoclonal antibodies to identify *Plasmodium* antigens [11]. Of the many antigens available malaria RDTs that target histidine rich protein 2 (HRP2), specific to *P. falciparum* have been widely used, due to its high sensitivity and heat stability [9, 12]. According to the 2023 World Malaria Report of the World Health Organization (WHO), for instance, the use of RDTs has increased with roughly 415 million RDTs based on HRP2 sold globally in 2022 [1].

PfHRP2 is synthesized during the asexual and early sexual stages of the life cycle of *P. falciparum* [13, 14], and shares structural similarities with PfHRP3, allowing antibodies directed against HRP2 to cross-react with HRP3 [14–17]. The PfHRP2 and PfHRP3 genes are located in the sub telomeric regions of chromosomes 8 and 13, respectively [18, 19]. They are characterized by variable tandem repeats, which are named as types based on the repeated motif [15, 20]. Each gene comprises two exons: exon 2 containing the main encoding sequence, and exons 1–2 which house signal and cleavage sequences [18].

HRP2-based RDTs have shown promise in improving malaria diagnosis in Ethiopia, even though the lower sensitivity reported recently from various endemic sites highlights the need for diagnostic performance monitoring [21, 22]. According to available reports, the extensive spread of *P. falciparum* that had deletion of PfHRP2 and PfHRP3 genes poses a threat to the use of HRP2-based RDTs in Ethiopia [21, 23–25]. Besides, the genetic diversity of parasites may play a role in the variation in these test kits performance [26–28]. The impact of genetic diversity in the PfHRP2 gene on the diagnostic performance of HRP2-based RDTs is still not well understood. Studies conducted elsewhere have demonstrated that variability does not substantially influence the performance of RDTs [16, 29–31]. On the other hand, there are studies that have reported the effects of PfHRP2's genetic diversity on RDT sensitivity, resulting in false negatives, especially at very low parasite densities (less than 200 parasites/μl) [32, 33].

Thus, monitoring the genetic diversity of PfHRP2 is crucial as it has the potential to compromise the diagnostic performances of RDT-based malaria diagnosis, thereby promoting timely and appropriate treatment of suspected patients. Limited data exist regarding PfHRP2 genetic variations and their effects on RDT performance, with only two studies having examined PfHRP2 diversity in samples from restricted geographic areas of Ethiopia in the past [19, 34].

The present study aimed to investigate the genetic diversity of PfHRP2 and its homologue PfHRP3 in uncomplicated malaria cases from three malaria-endemic sites in Ethiopia.

Methods

Study site and design

A cross-sectional health facility-based study was conducted between June 2022 and March 2023 at the Metehara, Zenzelema and Kolla Shelle health centres situated in malaria endemic sites in Ethiopia (Fig. 1). The study areas were selected to ensure geographical representation and to reflect the distribution of malaria transmission across three different ecological zones in Ethiopia. The Metehara Health Centre is in Metehara town, central Ethiopia, about 188 kms (Km) east of Addis Ababa, while the Zenzelema Health Centre is located 588 km northwest of Addis Ababa.

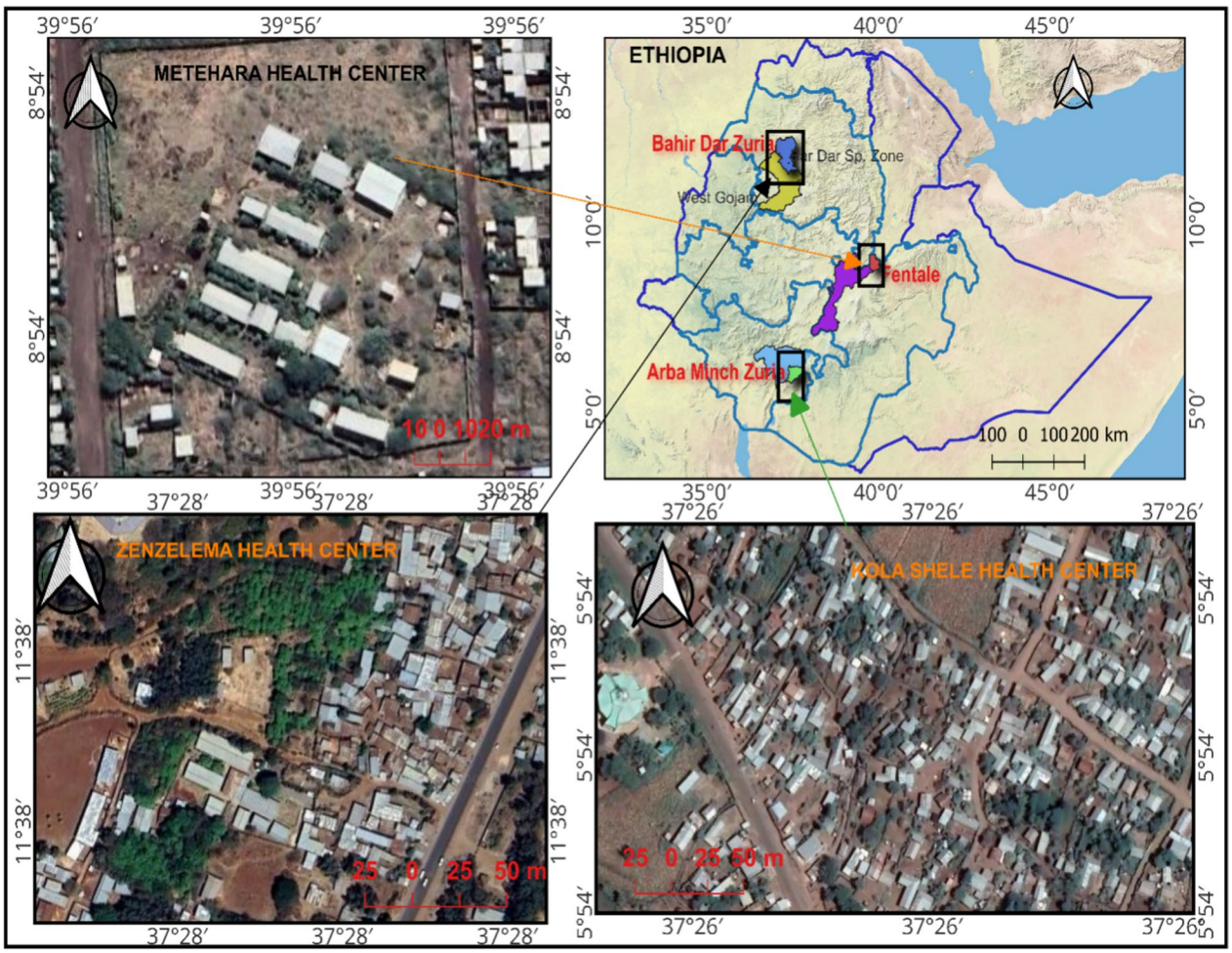

Fig. 1 Map of study sites in Ethiopia (generated using Quantum GIS version 3.36.0 software)

The Kolla-Shelle Health Centre is situated in the Arba Minch Zuria district, 532 kms southwest of Addis Ababa and roughly 27 kms from Arba Minch town. The towns of Metehara and Zenzelema experience year-round malaria transmission, with September to December being the peak month for transmission and April to May being the least [5, 35]. In contrast, the Kolla Shelle district experiences highly seasonal and unstable malaria transmission [36]. At each of the three study sites, *P. falciparum* was the most common parasite species, with prevalence rates of 17% in Metehara, 12.8% in Zenzelema, and 22.8% in Kolla Shelle [5, 35, 36]. Moreover, the study sites are all surrounded by water sources, which create ideal conditions for *Anopheles* mosquitoes to lay their eggs. The entomological inoculation rate (EIR) in Kolla Shelle is 17.1 infectious bites per person per year [37]. Unfortunately, the EIR for Metehara and Zenzelema has not been documented.

Study population and sample collection

The study populations were malaria-suspected patients, aged 5 years and older, who attended the selected health centres during the study period. Patients who had headaches, chills, sweating, and were febrile at the time of enrollment, or who had a history of a fever episode within the preceding 48 h, were screened. Participants with microscopically confirmed *P. falciparum* infections were purposely selected for enrollment. A written consent or assent was obtained before recruitment in the study. Patients with severe symptoms of malaria, such as seizures and decreased consciousness, were not included in the study.

Samples were obtained following demographic data collection and prior to initiation of anti-malarial therapy. Capillary blood samples from finger-pricks were taken from each study participant for microscopic examination and PfHRP2 based RDT testing. In addition, three ml of venous blood was aseptically drawn from each consented

participant, dispensed into EDTA tubes and stored at − 20 °C for molecular assays. The general workflow of the study is described and shown in Fig. 2.

Microscopy and malaria RDT

Thick and thin blood smears were stained with 10% Giemsa working solution for 10 min and examined under light microscopy. While thick smears were used to detect the presence of malaria infection and parasite density, thin smears were used to identify the species and stages of maturation of the parasites. A trained laboratory technician determined each microscopic examination in accordance with WHO guidelines [6]. Briefly, slides were examined by two independent microscopists, blinded each other across 200 high-power fields, assuming an average WBC count of 8,000/µl. In cases of discrepancy, a third independent blinded reading was conducted. Final parasitaemia values were determined using the Obare Method Calculator [38].

SD Bioline™ Malaria RDTs (Pf/Pv HRP2/PvLDH) with product code 05FK80 was used to detect *P. falciparum* infections. All RDT tests were performed and interpreted

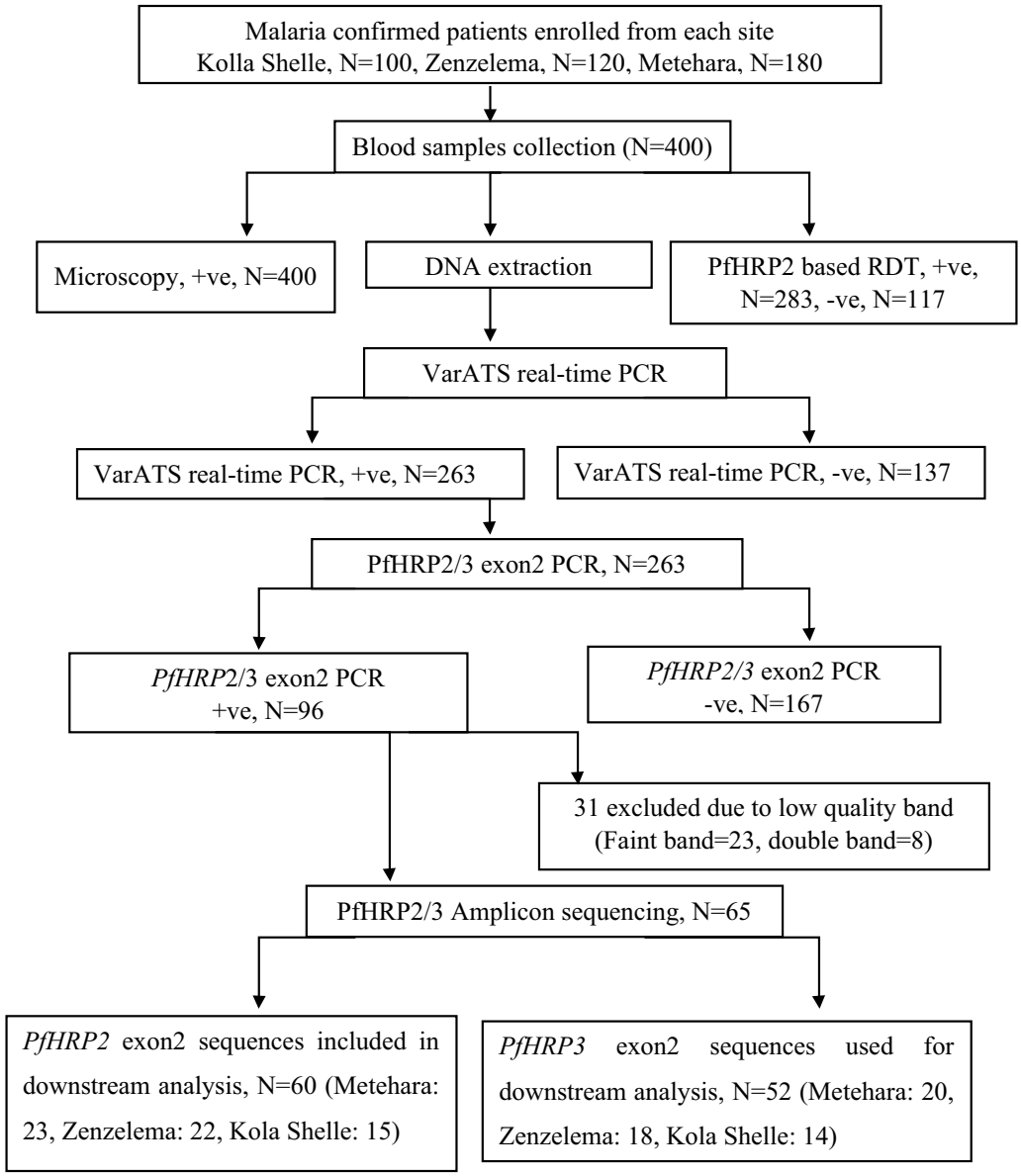

Fig. 2 Study flow chart that shows the overall study procedures for PfHRP2 and 3 genetic diversity analysis**,** Ethiopia. *Var*ATS: *var* gene acidic terminal sequence PCR, – Ve: negative, + Ve: Positive, N: number of samples

by trained laboratory personnel from the selected health centres following the manufacturer's instructions.

Genomic DNA extraction and P. falciparum confirmation

Parasite DNA was extracted from 200 µl of whole blood samples using a G-spin™ total DNA extraction mini kit according to the manufacturer's instructions. The extracted DNA was subsequently eluted in 50 µl elution buffer, stored at -20^{0}C and transported to The Medical Research Council Unit the Gambia at London School of Hygiene and Tropical Medicine (MRCG@LSHTM) for molecular analysis. *P. falciparum* identification was confirmed by *var* gene acidic terminal sequence (*varATS*) real-time PCR using the Bio-Rad CFX96 real-time PCR machine as previously described [39]. A cutoff threshold (Ct) value of 40 was used to distinguish positive and negative samples.

Pfhrp2 and pfhrp3 genes amplicon sequencing

All PCR confirmed *P. falciparum* positive samples were included for *pfhrp2/3* amplicon sequencing. Amplifications of *pfhrp2* and *pfhrp*3 exon2 genes were performed using primers described by Baker et al. [28]. Briefly, 5 µl of DNA template was added into a master mix containing 1 × ThermoPol reaction buffer (New England BioLab, USA), 0.2 mM dNTP (New England BioLab, USA), 0.1U/µl of Taq DNA polymerase (New England BioLab, USA), and 0.2 µM of each primer (Forward 1(F1) and Reverse 2 (R2) as shown in Table 1) in a total PCR reaction volume of 25 µl. Genomic DNA of three *P. falciparum* strains were used as controls, namely 3D7 (*pfhrp2* + and *pfhrp3* +), Dd2 (*pfhrp2* − and *pfhrp3* +) and Hb3 (*pfhrp2* + and *pfhrp3* −). The amplifications were performed under the following PCR conditions: 5 min at 95 °C initial denaturation, followed by 39 cycles of 95 °C denaturation for 45 s, 50 °C annealing for 1 min, 68 °C extension for 1 min, and a final extension at 70 °C for 5 min. The PCR products of *pfhrp2* exon2 and *pfhrp3* exon2 were visualized on a 2% agarose gel electrophoresis using a 100 bp DNA ladder to identify the DNA bands and confirm the expected amplicon size of 600–650 bp. Low-quality bands that appeared faint or as double bands were excluded from further analysis.

Table 1 Primer assays and sequences used to amplify *pfhrp*2/3 exon2 genes

Primer name	Target gene	Sequence (5′–3′)
Pfhrp-2 exon2 F1	Pfhrp-2 exon2	CAA AAG GAC TTA ATT TAAATA AGA G
Pfhrp-2 exon2 R2	Pfhrp-2 exon2	AAT AAA TTT AAT GGC GTA GGC A
Pfhrp-3 exon2 F1	Pfhrp-3 exon2	AAT GCA AAA GGA CTT AAT TC
Pfrp-3 exon 2 R2	Pfhrp-3 exon2	TGG TGT AAG TGA TGC GTA GT

The generated amplicons were pooled, and sequencing libraries were prepared using the SQK-109 with EXP-NBD196 ligation sequencing kits from Oxford Nanopore Technologies, following the manufacturer's instructions. The sequencing was conducted on the GridION using the FLO-MIN114 Flow cell. The entire process lasted 72 h, yielded 64.04 GB of data, 4.81 Gb of bases, 8.15 M reads, and 3.67 Gb pass bases. For Base calling and demultiplexing, minimum barcoding quality was set to 60, minimum read quality score was set to 9, and no minimum read length filter was set. Base-calling and barcoding was performed by guppy v. 7.0.9 within MinKNOW v23.07.5.

Consensus sequence generation

Briefly, following quality control analysis and barcode trimming, sequence reads were aligned to the reference genomic sequences for each gene; HRP2 (NC_004329.3) and HRP3 (NC_004331.3) target using minimap2(version 2.21-r1071. Samtools (version 1.8) was used to sort and convert the output to binary alignment map (BAM) file. In addition, sequence reads were also mapped to the full 3D7 reference genome (PlasmoDB PF3D7_0831800 and PlasmoDB PF3D7_1372200) and manually inspected using the Integrative Genomics Viewer (IGV) (version 2.3.94) tool, to confirm the presence of reads at expected gene coding location on chromosomes. The FASTQ files that successfully aligned to target genes were used as input for de novo assembly. Consensus sequences were produced using Canu, Geneious contigs (version 2023.2.1) and Amplicon sorter, a tool for building reference-free consensus sequences using ONT-sequenced amplicons based on read similarity and length. The Canu, Amplicon sorter and Geneious contigs were all converted to consensus sequences, visually explored, and manually edited to fit in the correct translation frame. Up to ten contig consensus sequences per sample were curated based on their having > 10 reads and few low-quality bases. Therefore, consensus was generated following majority calls. To align the PfHRP2/3 gene sequences to their respective reference genomes, the minimap2 tool with the "map-ont" option was used. The nanopore reads covered 75% of the 1064 bp HRP2 (PF3D7_0831800) and the 977 bp HRP3 F3D7_1372200 reference genomes at a 100% mapping rate. Finally, consensus sequences were successfully generated for HRP2 in 60/65 isolates and HRP3 in 52/65 isolates. The nucleotide sequences were then translated to amino acid sequences and exported to FASTA file format. Amino acid repeat type counts were calculated and annotated using Geneious as described previously [16, 28].

Data analysis

Descriptive statistics were employed using Stata software version 14 (Stata Corporation, TX, USA) to determine the frequency, occurrence, and structural organization of amino acid repeat type. PfHRP2 and PfHRP3 genetic diversity was described using numerically coded repeat types 1 to 24 as reported by Baker et al. [16, 28]. The Chi-square and Fisher's exact tests were used to determine if there was a statistically significant relationship in the distributions of PfHRP2 type repeats among the study sites, with $P < 0.05$ considered significant. *Plasmodium falciparum* isolates were categorized into four groups according to Baker et al. [16, 28] model, based on the product of the number of PfHRP2 repeat types 2 and 7 (type 2 × type 7). These groups were A (very sensitive), B (sensitive), I (borderline), and C (non-sensitive) when the number of type 2 × type 7 was above 100, ranged from 50 to 100, ranged from 44 to 49, and less than 43, respectively.

Results

Out of the 263 *P. falciparum* PCR-confirmed clinical samples, 96 successfully generated amplicons for exon2 of *PfHRP2* and *PfHRP3*. Thirty one out of the 96 amplicons showed poor bands on the agarose gel electrophoresis, which excluded them from further analysis. Amplicons of 65 PfHRP2 and PfHRP3 were selected, library prepared and sequenced using Oxford Nanopore technology (ONT) to assess their genetic diversity. The PfHRP2/3 nucleotides generated by this study were uploaded to the NCBI Genebank (accession numbers: PP662729-PP662788 for PfHRP2 and PP662789-PP662840 for PfHRP3).

Pfhrp2 genetic diversity

A total of 60 (92.3%) samples with *PfHRP2* exon 2 amplicons produced quality read sequences and were used for consensus generations and downstream analysis. Sequencing analysis showed that the amplified *PfHRP2* exon *2* fragments ranging from 495 to 792 base pair and composed of amino acids residues ranging from 186 to 289. A total of 12 previously reported *PfHRP2* amino acid repeat types were identified in the *P. falciparum* isolates (Table 2). However, these amino acid repeat types varied in composition, number, and order, resulting in wide variations in the size of PCR fragments. For each sample, *PfHRP2* had a range of 20 to 32 distinct types of amino acid repeats.

The patterns of amino-acid repeat organization in the sequences were similar, albeit with minor differences. The sequence patterns found in the study were categorized into patterns (I-IX) as illustrated in Fig. 3. No single sequence pattern type was found to be unique, with each type occurring more than once. Every sequence had a type 1 repeat type (AHHAHHVAD) at the start and a type 12 repeat type (AHHAAAHHEAATH) at the end (Fig. 3). The most common repeats among isolates were types 2 and 7, which were widely dispersed among all the PfHRP2 amino acid sequences. Furthermore, types 1, 8, 10, and 12 repeats were present in every isolate with moderate frequencies ranging from 1 to 5 times per isolate, whereas repeat types 3, 4, 5, and 6 were identified in 60% to 85% of Pfhrp2 amino acid sequences. Repeat types 13 and 14 were infrequent, appearing in only two isolates, accounting for 3.3% of the total (Table 2). Further sequence analysis did not identify any novel amino acid repeats in any of the PfHRP2 sequences.

The frequency distributions of PfHRP2 type repeats within the study sites indicate that there is no significant variation in the occurrence of different repeat types across the three study sites ($P > 0.005$) (Table 3). Apart from repeat types 13 and 14, which were only detected in isolates collected from Zenzelema Health Centre, the majority of PfHRP2 repeat types had comparable occurrences both within and between study sites. The occurrences of PfHRP2 repeat types in the study sites are displayed in (Table 3).

PfHRP3 genetic diversity

A total of 52 (80%) sequences for *PfHRP3* exon2 amplicons yielded quality data was used for further analysis. The length of *Pfhrp3* exon 2 varied from 515 to 695 base pairs, corresponding to a protein composed of 171 to 231 amino acids. Additionally, the total number of amino acid repeat types per isolate ranged between 23 and 30 types. Eight previously documented amino acid repeat types were found among the 52 isolates (Table 2). PfHRP3 had a more diverse structural organization of repeat type than PfHRP2. Twelve different patterns of PfHRP3 were identified in more than one isolate (Fig. 4). Type 1 repeats (AHHAHHVAD) were located at the start of every sequence, and 92.3% of them ended with a type 4 repeat (AHH), with the remaining 7.7% ending with repeat type 17. More importantly, a non-repeat (ANHGFHF−−NLHDNNSHT) sequence that falls between the type 7 and type 20 amino acid repeat type was present in all isolates (Fig. 4).

Repeat types 1, 4, and 7, which were identified in PfHRP2, were also found in PfHRP3 but at lower frequencies per isolate compared to PfHRP2. The PfHRP3 sequence lacks the type 9, 11, and 19 repeat types, like the PfHRP2 sequence (Table 2). Moreover, in the present study, repeat types 21, 22, 23, and 24 were not found in either of the amino acid sequences.

All PfHRP3 sequences contained repeat type 16, with the most repeated type occurring 11–18 times per

Table 2 Distribution of PfHRP2 and PfHRP3 Amino acids repeat in *P. falciparum* isolates from malaria endemic area, Ethiopia

Repeat type	Amino acid repeat sequence*	PfHRP2 (no = 60)		PfHRP3 (no = 52)	
		No (%)	Min to Max number	No (%)	Min to Max number
1	AHHAHHVAD	60 (100)	1–5	52 (100)	1–4
2	AHHAHHAAD	60 (100)	4–13	0 (0)	0
3	AHHAHHAAY	52 (86)	0–2	0(0)	0
4	AHH	41 (68)	0–2	51 (98)	0–1
5	AHHAHHASD	50 (83)	0–2	0 (0)	0
6	AHHATD	36 (60)	0–5	0 (0)	0
7	AHHAAD	60 (100)	3–11	50 (96)	0–1
8	AHHAAY	60 (100)	1–2	0 (0)	0
9	AAY	0 (0)	0	0 (0)	0
10	AHHAAAHHATD	60 (100)	1–2	0 (0)	0
11	AHN	0 (0)	0	0 (0)	0
12	AHHAAAHHEAATH	60 (100)	1	0 (0)	0
13	AHHASD	2 (3.3)	0–1	0 (0)	0
14	AHHAHHATD	2 (3.3)	0–1	0 (0)	0
15	AHHAHHAAN	0 (0)	0	34 (65)	0–1
16	AHHAAN	0(0)	0	52 (100)	11–18
17	AHHDG	0 (0)	0	52 (100)	4–8
18	AHHDG	0 (0)	0	52 (100)	2–4
19	AHHAA	0 (0)	0	0(0)	0
20	SHHDD	0 (0)	0	52 (100)	1
21	AHHAHHATY	0 (0)	0	0 (0)	0
22	AHHAHHAGD	0 (0)	0	0 (0)	0
23	ARHAAD	0 (0)	0	0 (0)	0
24	AHHTHHAAD	0 (0)	0	0 (0)	0

N: total number of PfHRP2 and PfHRP3 amino acid sequence in *P. falciparum* isolates, Min: minimum number of amino acid repeat, Max: maximum number of amino acid repeat

Asterisk (*) indicate amino acid repeat type of PfHRP2 and PfHRP3 as described [16, 28]

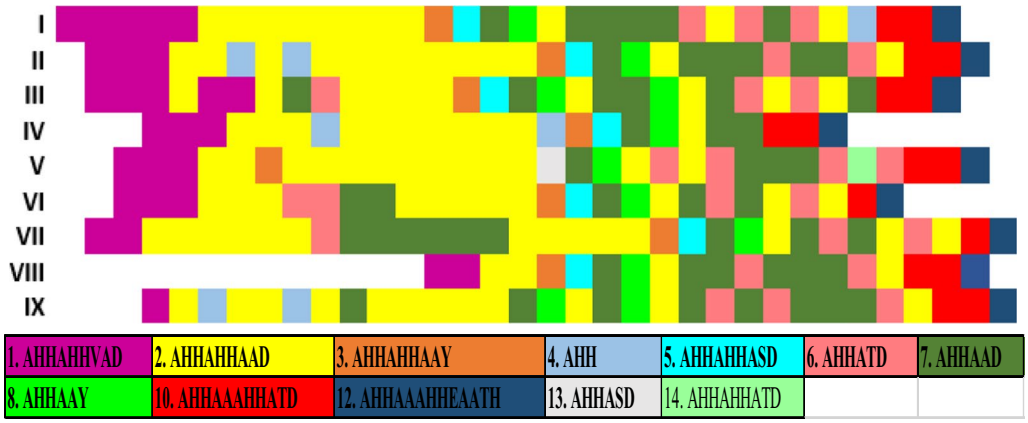

Fig. 3 Patterns of PfHRP2 sequences of *P. falciparum* isolates from malaria endemic site, Ethiopia. PfHRP2 sequence patterns I to IX were identified in the current study. Colours distinguish the twelve PfHRP2 amino acid repeat type found in this study. Amino acid repeat types were identified as previously described Baker et al. [16, 28]

Table 3 Comparison of the occurrence of individual PfHRP2 repeat types within the three health centres in Ethiopia

Type of repeat	Amino acid repeat sequence	Zenzelema (22) No (%)	Metehara (23) No (%)	Kola Shelle (15) No (%)	P. value
1	AHHAHHVAD	22 (100)	23 (100)	15 (100)	ns
2	AHHAHHAAD	22 (100)	23 (100)	15 (100)	ns
3	AHHAHHAAY	20 (91)	19 (82.6)	13 (86.6)	ns
4	AHH	15 (68)	16 (69.5)	10 (66.6)	ns
5	AHHAHHASD	18 (81.8)	20 (86.9)	12 (80)	ns
6	AHHATD	14 (63.6)	11 (47)	11 (73.3)	ns
7	AHHAAD	22 (100)	23 (100)	15 (100)	ns
8	AHHAAY	22 (100)	23 (100)	15 (100)	ns
10	AHHAAAHHATD	22 (100)	23 (100)	15 (100)	ns
12	AHHAAAHHEAATH	22 (100)	23 (100)	15 (100)	ns
13	AHHASD	2 (9)	0 (0)	0 (0)	ns
14	AHHAHHATD	2 (9)	0 (0)	0 (0)	ns

Statistically significant difference ($p < 0.05$)

ns: non-significant

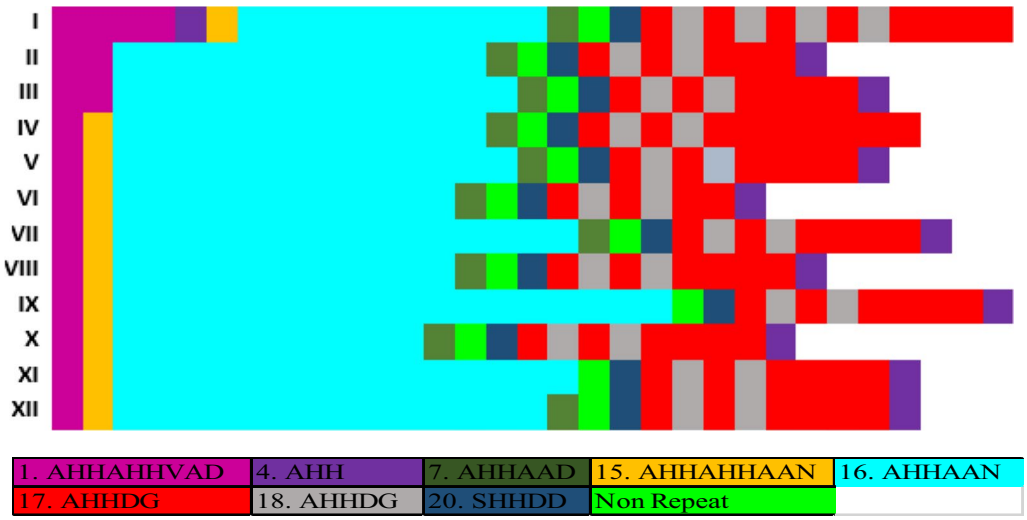

Fig. 4 PfHRP3 structural organization from malaria endemic site Ethiopia. Thirteen (I-XII) PfHRP3 patterns identified in more than one isolates. Colours distinguish the eight PfHRP3 amino acid repeat type found in this study. Amino acid repeat types were identified as previously described Baker et al. [16, 28]

isolate. Likewise, all samples contained repeat types 1, 17, 18, and 20 in PfHRP3, but these repeat types exhibited significantly fewer repeats per sequence. Repeat type 15 occurs less frequently than other repeat types in only 65% of the isolates. Furthermore, in six isolates (11.5%), the study found a novel variant repeat type 17 V (SHHDG) that has never been documented (reported) in Ethiopia.

Prediction of the sensitivity of PfHRP2-based RDTs using Bakers' logistic regression model

The relationship between number and combination of major repeat types 2 and 7 was investigated to assess the impact of PfHRP2 genetic diversity on RDTs diagnostic performances. Based on PfHRP2 diversity, the study categorized *P. falciparum* isolates into groups A, B, I, and C by multiplying the number of repeats of type 2 and type 7 (type 2 × type 7). The analysis showed that most of the isolates contained group C (51.6%) followed by group B (38.3%). Group A and I were the least frequent, accounted for 5% of the HRP2 sequences sequenced (Table 4). Interestingly, PfHRP2-based RDT detected 100% (3/3) of the isolates in group A and 85.7% (18/21) in group B at a parasitaemia level > 250 parasite per microlitre of blood. Nonetheless, for isolates categorized

Table 4 Frequency of PfHRP2 sequence groups based on the Baker's model and PfHRP2 based RDT results

Group	Frequency (N = 60) No (%)	PfHRP2 RDT Positivity No (%)	Microscopy > 250 parasite/µl No (%)
Group A	3 (5)	3 (100)	3 (100)
Group B	23 (38.3)	18 (85.7)	21(91.3)
Group I	3 (5)	2 (66.6)	3 (100)
Group C	31 (51.6)	26 (86)	28 (90.3)

PfHRP2 sequence isolates were categorized into four groups based on the frequency of types 2 × type 7 repeats: group A (≥ 100, highly sensitive), group B (50–99, sensitive), group C (< 43, non-sensitive), and group I (44–49, borderline)

under group C, no association was found between the length of these two type repeats and the PfHRP2-based RDTs' sensitivity. As illustrated in Table 4, 26 of the 28 group C sequences that the Baker model classified as non-sensitive were found to be sensitive to PfHRP2 RDT.

Discussion

Successful malaria control relies on an efficient diagnostic tool to identify cases before initiating anti-malarial treatment in accordance with WHO guidelines [40]. Malaria RDTs were introduced in Ethiopia in 2004 and have become widely valued diagnostic tools for resource limited settings, where a large burden of malaria occurs [3, 10]. In Ethiopia, RDT targeting PfHRP2 are extensively used, but the spread of isolates that do not express PfHRP2/3 genes [23–25] is a threat to their efficacy and continued use. Besides, the impact of PfHRP2 genetic diversity on PfHRP2 based RDT performance is not well understood in the country. Analysing the PfHRP2/3 genetic diversity is crucial for public health, as it enables the monitoring of RDT effectiveness.

The current study provides significant insights into the genetic variations of PfHRP2 and PfHRP3 across three health centres in malaria-endemic areas in Ethiopia. The structural organization of PfHRP2/3 showed minor variations in the frequency and number of amino acid repeat types per isolate across the three health centres. Comparable findings have been found in earlier studies carried out in Western Ethiopia [34], the Brazilian Amazon [41] and Kenya [42], with PfHRP2 and 3 repeat types being present at consistent frequencies throughout malaria-endemic areas. Conversely, wide variations were found in the number and frequency of repeat types in the PfHRP2 and 3 amino acid sequences of clinical *P. falciparum* isolates collected from various regions of India [30].

Twelve different amino acid repeat types were identified in the PfHRP2 sequences of isolates and these findings are consistent with what has also been reported for Yemen [29]. Two previous studies conducted in Ethiopia have documented PfHRP2 amino acid repeat types of eleven and fifteen, respectively [19, 34]. According to the study's findings, every *P. falciparum* isolate examined had PfHRP2 amino acid sequences that started with a type 1 repeat and ended with a type 12 repeat. The results of this study align with earlier research that showed that most PfHRP2 repeats start with type 1 and conclude with type 12 repeats [29, 30, 42]. In contrast, discrepancies were reported against earlier studies done in Ethiopia, where the majority of PfHRP2 sequences ended with a type 10 repeat and only 10% terminated with a type 12 repeat [34]. It could be possible that over time the type 12 isolates have been selected for and became dominant.

The current study also reveals repeat types 2 and 7 were consistently found in all PfHRP2 amino acid sequences, while repeat types 9 and 11 were entirely absent in every isolate, which is consistent with other research [29, 34, 42–44]. However, repeat type 9 were reported recently in a small proportion of infections from northern Ethiopia [19] and Senegal [20], and repeat type 11 were reported from Madagascar *P. falciparum* clinical isolates [31]. Types 1, 8, 10, and 12 were the most common repeats in PfHRP2, while types 13 and 14 were rare repeats that accounted for 3.3% of all amino acid sequences. These findings align with earlier studies [28, 30, 44]. Type 4 repeats were found in 40% of the isolates, which is also comparable to the findings of the earlier study from western Ethiopia [34]. The wide variation of PfHRP2 amino acid repeat types observed across the studies might be due to extensive deletion of the HRP2 gene reported globally [45, 46], which was followed by clonal expansion and diversification [14, 47].

The present study showed that the sequenced isolates from PfHRP3 displayed low genetic variation in terms of the number and frequency of amino acid repeat type when compared to PfHRP2 as has been previously reported [19, 34, 42]. The eight identified amino acid sequences in the current study were repeat types 1, 4, 7, 15, 16, 17, 18, and 20 and these are like previously reported from global isolates [25] and western Ethiopia [34]. Apart from the eight amino acid repeat types identified in the current study, repeat type 19, with a lower frequency of 1.25%, was reported from Northern Ethiopia [19].

Interestingly, 100% of PfHRP3 sequences begin with type 1 repeat, either one or more copies, and the majority ended with type 4 repeat, which is consistent with other studies reports [30, 34, 42]. Moreover, every isolate of the PfHRP3 amino acid sequence contained a non-repeating sequence that falls between the type 7 and type 20 amino acid repeat type, as shown before [28, 34, 42]. In line with previous findings from Ethiopia [34] and Kenya

[42], type 16 amino acid repeat was the most prevalent and frequently identified type in this investigation. The widespread presence of these repeat type might contribute to increased antigenicity and imply that it might be useful for RDT detection [19]. Furthermore, strong correlation was reported between the numbers of PfHRP3 repeats 16 and 17 and the sensitivity of PfHRP2-based RDT [34].

The present study showed that PfHRP2 doesn't contain any novel repeat type; but 11.5% of PfHRP3 sequences had novel repeat types 17 V, not previously reported in Ethiopia. These had however been seen in isolates from India [30], Kenya [42] and the China-Myanmar border [44]. A larger nation-wide study may determine if they are common in the country or occurred recently de novo or by importation. Novel PfHRP2 and PfHRP3 repeat types are as a result of substitutions of a single or multiple amino acid in the repeat types [30, 43]. The rare occurrences of novel repeat types in the current study, when compared to two earlier studies [19] and [34], which were carried out, respectively, in northern and western parts of Ethiopia, suggest that geographic differences may be important in the diversity of PfHRP2 and PfHRP3 genes.

The study found that repeat types 1, 4, and 7, were detected in both PfHRP2 and PfHRP3 amino acid sequences consistent with another finding [34]. Besides, these amino acid repeat, type 2 repeat was identified in both PfHRP2 and PfHRP3 amino acid sequences of isolate from Indian [30] and Kenyan [42]. The structural similarity between the two amino acid repeat type is responsible for the cross-reactivity of the monoclonal antibodies specific for PfHRP2 against PfHRP3 [15, 48, 49]. PfHRP3 may be helpful in reducing the effects of PfHRP2 antigenic polymorphism and may be contributing to the diagnosis of malaria using PfHRP2-based rapid diagnostic tests, according to earlier research [15, 48].

The impact of genetic variations on the diagnostic performance of PfHRP2 based RDTs, is not well understood. Baker et al. [28] used a logistic regression model to demonstrate how the product of repeat types 2 and 7 affects the sensitivity of PfHRP2-based RDTs, by using samples from culture-adapted *P. falciparum*. According to the model, at parasite densities of less than 250 parasites/µl, a combined product of repeat types 2 and 7 (type 2×type 7) is classified as group A (very sensitive), 50 to 100 as group B (sensitive), 44 to 49 as group I (borderline), and less than 43 as group C (non-sensitive) [28]. Later, Baker et al. disproved this finding by utilizing *P. falciparum* clinical isolates collected from various geographic areas and failing to correlate repeat length polymorphism to HRP2-based RDT sensitivity [16]. Likewise, studies in other malaria endemic regions have shown that genetic variability has no appreciable effect on RDT performance [30, 31, 50]. Conversely, few studies concur with Baker's regression model, especially group C (type 2×type 7; ranges < 43), was associated with RDT lower detection and false negativity [32, 33].

In the current study, most *P. falciparum* clinical isolates were classified in group C, whereas previous studies conducted in African countries including Ethiopia found that the majority of isolates were in group B [20, 31, 34, 42]. The relationship between number and combination of major repeat types 2 and 7 on PfHRP2-based RDT sensitivity was examined in this study and found that most of the isolates belong to groups A and B were predicated to be detected by PfHRP2 RDT at parasitaemia level > 250 parasite/µl. The finding is in line with study done in Ethiopia [34], Senegal [20], Madagascar [31]. Additionally, PfHRP2 RDT also detected most *P. falciparum* isolates in group C at a parasitaemia level of ≥ 250 parasites/µl, in agreement with earlier findings [30, 34]. The study finding contradict with the Baker' model et al. [16], which states that PfHRP2-based RDT is not sensitive if the length of repeat types 2 and 7 is less than 43 (group C).

Limitation of the study

Despite having surveyed representative samples from each study site, limited number of samples underwent successful PfHRP2 and PfHRP3 amplification as a result of widespread gene deletion in Ethiopia. This limited number hinders further generalization, as this may have also limited the amount of diversity captured. Moreover, Nanopore sequencing technology offers great advantages through its capability to sequence long stretches of repeat regions of PfHRP2/3. However, it has a higher base-level error rate, which impacts downstream analyses such as de novo assembly. Besides, the effect of polyclonal infections on the accuracy of reads is still not known and will require further assessment.

Conclusion

The current study showed, PfHRP2 and 3 are highly diverse among the Ethiopian *Plasmodium falciparum* clinical isolates, as evidenced by the variety of amino acid repeat types and their structural arrangement. A novel repeat that had never been reported in Ethiopia was found in the amino acid sequences of PfHRP3. In addition, the study also highlighted that most isolates in the present investigation are categorized as belonging to group C in accordance with the Baker model and they are sensitive for PfHRP2-based RDTs. Continuous monitoring of the effectiveness of PfHRP2-based RDTs and diversity of the repeat type in PfHRP2/3

across different malaria endemic ecological settings is necessary for malaria elimination.

Abbreviations

RDT	Rapid diagnostic tests
HRP2/3	Histidine-Rich Protein 2/3
pfhrp2/3gene	Plasmodium falciparum Histidine-rich protein 2 and 3 genes
PCR	Polymerase chain reaction
*Var*ATS	*var* Gene acidic terminal sequence
NCBI	National Centre for Biotechnology Information's
ONT	Oxford Nanopore sequencing technology
QC	Quality Check
IRB	Institute Review Board
DNA	Deoxyribonucleic acid

Acknowledgements
Authors sincerely thank the Medical Research Council Unit the Gambia, London School of Hygiene and Tropical Medicine, for granting access to their molecular biology lab, and PAMGEN H3Africa project for financial support. Additionally, we appreciate study participants for their voluntary participation. We extend our special gratitude to the laboratory technician and field data collectors who took part in the research.

Author contributions
LG and AM conceived and designed the study. AM and BM collected samples. FC, AM, BM, EO, MK, BE, and AAN conducted laboratory analysis. AMK and AM performed data analysis. AM drafted the original manuscript. AMK, AAN, LG and EO reviewed and edited the manuscript. All authors read and approved the final manuscript.

Funding
The PAMGEN H3 Africa project (H3A/002/18) and Addis Ababa University post graduate studies partly funded for this work. The funders had no role in the study design, data collection, reporting of this study.

Availability of data and materials
The data sets analysed during the current study are available from the corresponding author on reasonable request. *Plasmodium falciparum* histidine-rich proteins 2 and 3 nucleic acid sequences are available in GenBank (PfHRP2 accession numbers: PP662729-PP662788 and PfHRP3 accession numbers PP662789-PP662840).

Declarations

Ethics approval and consent to participate
The study received approval from the Institutional Review Board (IRB) of the Aklilu Lemma Institute of Pathobiology, Addis Ababa University (Ref No ALIPB/ IRB77/2014/22). Blood samples were collected after obtaining written informed consent and/or assent from parents or guardian for children. Patients in the study who had been diagnosed with malaria were treated by medical professionals in accordance with national treatment guidelines.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Author details
[1]Aklilu Lemma Institute of Pathobiology, Addis Ababa University, Addis Ababa, Ethiopia. [2]College of Natural and Computational Science, Hawassa University, Hawassa, Ethiopia. [3]Malaria and NTDs Research Team, Bacterial, Parasitic, and Zoonotic Diseases Research Directorate, Ethiopian Public Health Institute, Addis Ababa, Ethiopia. [4]Medical Research Council Unit the Gambia, London School of Hygiene and Tropical Medicine, Banjul, The Gambia.

Received: 23 April 2024 Accepted: 22 August 2024
Published online: 29 August 2024

References

1. WHO. World malaria report 2023. Geneva: World Health Organization; 2023.
2. Jagannathan P, Kakuru A. Malaria in 2022: increasing challenges, cautious optimism. Nat Commun. 2022;13:2678.
3. FMOH. National Malaria Elimination Strategic plan 2021–2025. Addis Ababa: FMOH; 2020.
4. FMOH. National malaria strategic plan 2017–2020. Addis Ababa: FMOH; 2017.
5. Mandefro A, Tadele G, Mekonen B, Golassa L. Analysing the six-year malaria trends at Metehara Health Centre in Central Ethiopia: the impact of resurgence on the 2030 elimination goals. Malar J. 2024;23:32.
6. WHO. Global technical strategy for malaria 2016–2030. Geneva: World Health Organization; 2016.
7. Wongsrichanalai C, Barcus MJ, Muth S, Sutamihardja A, Wernsdorfer WH. A review of malaria diagnostic tools: microscopy and rapid diagnostic test (RDT). Am J Trop Med Hyg. 2007;77:119–27.
8. Oyegoke OO, Maharaj L, Akoniyon OP, Kwoji I, Roux AT, Adewumi TS, et al. Malaria diagnostic methods with the elimination goal in view. Parasitol Res. 2022;21:1867–85.
9. Martiáñez-Vendrell X, Skjefte M, Sikka R, Gupta H. Factors affecting the performance of HRP2-based malaria rapid diagnostic tests. Trop Med Infect Dis. 2022;7:265.
10. EPHI. Ethiopian public health institute. Services availability and readiness assessment. Addis Ababa: EPHI; 2018.
11. Maltha J, Gillet P, Jacobs J. Malaria rapid diagnostic tests in travel medicine. Clin Microbiol Inect. 2013;19:408–15.
12. WHO. Malaria rapid diagnostic test performance: results of WHO product testing of malaria RDTs: round 8 (2016–2018). Geneva: World Health Organization; 2018.
13. Desakorn V, Dondorp AM, Silamut K, Pongtavornpinyo W, Sahassananda D, Chotivanich K, et al. Stage dependent production and release of histidine-rich protein 2 by *Plasmodium falciparum*. Trans R Soc Trop Med Hyg. 2005;99:517–24.
14. Baker J, Gatton ML, Peters J, Ho MF, McCarthy JS, Cheng Q. Transcription and expression of *Plasmodium falciparum* histidine-rich proteins in different stages and strains: implications for rapid diagnostic tests. PLoS ONE. 2011;6: e22593.
15. Lee N, Baker J, Andrews KT, Gatton ML, Bell D, Cheng Q, et al. Effect of sequence variation in *Plasmodium falciparum* histidine-rich protein 2 on binding of specific monoclonal antibodies: implications for rapid diagnostic tests for malaria. J Clin Microbiol. 2006;44:2773–8.
16. Baker J, Ho M, Pelecanos A, Gatton M, Chen N, Abdullah S, et al. Global sequence variation in the histidine-rich proteins 2 and 3 of *Plasmodium falciparum*: implications for the performance of malaria rapid diagnostic tests. Malar J. 2010;9:129.
17. Kong A, Wilson SA, Ah Y, Nace D, Rogier E, Aidoo M. HRP2 and HRP3 cross-reactivity and implications for HRP2-based RDT use in regions with *Plasmodium falciparum* hrp2 gene deletions. Malar J. 2021;20:207.
18. Poti KE, Sullivan DJ, Dondorp AM, Woodrow CJ. HRP2: transforming malaria diagnosis, but with caveats. Trends Parasitol. 2020;36:112–26.
19. Molina-de la Fuente I, Yimar M, García L, González V, Amor A, Anegagrie M, et al. Deletion patterns, genetic variability and protein structure of pfhrp2 and pfhrp3: implications for malaria rapid diagnostic test in Amhara region, Ethiopia. Malar J. 2022;21:287.
20. Deme AB, Park DJ, Bei AK, Sarr O, Badiane AS, Gueye P, et al. Analysis of pfhrp2 genetic diversity in Senegal and implications for use of rapid diagnostic tests. Malar J. 2014;13:34.
21. Mekonnen B, Dugassa S, Feleke SM, Taddesew BD, Tegegn BG, Adamu A, et al. Widespread pfhrp2/3 deletions and HRP2-based false-negative results in southern Ethiopia. Malar J. 2024;23:108.
22. Alemayehu GS, Lopez K, Dieng CC, Lo E, Janies D, Golassa L. Evaluation of PfHRP2 and PfLDH malaria rapid diagnostic test performance in Assosa Zone, Ethiopia. Am J Trop Med Hyg. 2020;103:1902–9.
23. Feleke SM, Reichert EN, Mohammed H, Brhane BG, Mekete K, Mamo H, et al. *Plasmodium falciparum* is evolving to escape malaria rapid diagnostic tests in Ethiopia. Nat Microbiol. 2021;6:1289–99.
24. Alemayehu GS, Blackburn K, Lopez K, Dieng CC, Lo E, Janies D, et al. Detection of high prevalence of *Plasmodium falciparum* histidine-rich protein 2/3 gene deletions in Assosa zone, Ethiopia: implication for malaria diagnosis. Malar J. 2021;20:109.

25. Golassa L, Messele A, Amambua-Ngwa A, Swedberg G. High prevalence and extended deletions in *Plasmodium falciparum* hrp2/3 genomic loci in Ethiopia. PLoS ONE. 2020;15: e0241807.
26. Hamid MA, Awad-Elgeid M, Nasr A. Gene variation and suspected *Plasmodium falciparum* histidine-rich protein 2 gene deletion and its impact on sensitivity of malaria rapid diagnostic tests in Sudan. BMJ Glob Health. 2017;2:A21.
27. Amoah LE, Abankwa J, Oppong A. *Plasmodium falciparum* histidine rich protein-2 diversity and the implications for PfHRP 2: based malaria rapid diagnostic tests in Ghana. Malar J. 2016;15:101.
28. Baker J, McCarthy J, Gatton M, Kyle DE, Belizario V, Luchavez J, et al. Genetic diversity of *Plasmodium falciparum* histidine-rich protein 2 (PfHRP2) and its effect on the performance of PfHRP2-based rapid diagnostic tests. J Infect Dis. 2005;192:870–7.
29. Atroosh WM, Al-Mekhlafi HM, Al-Jasari A, Sady H, Al-Delaimy AK, Nasr NA, et al. Genetic variation of pfhrp2 in *Plasmodium falciparum* isolates from Yemen and the performance of HRP2-based malaria rapid diagnostic test. Parasit Vectors. 2015;8:388.
30. Kumar Bharti P, Singh Chandel H, Krishna S, Nema S, Ahmad A, Udhayakumar V, et al. Sequence variation in *Plasmodium falciparum* histidine rich proteins 2 and 3 in Indian isolates: implications for malaria rapid diagnostic test performance. Sci Rep. 2017;7:1308.
31. Willie N, Mehlotra RK, Howes RE, Rakotomanga TA, Ramboarina S, Ratsimbasoa AC, et al. Insights into the performance of SD Bioline Malaria Ag Pf/Pan rapid diagnostic test and *Plasmodium falciparum* histidine rich protein 2 gene variation in Madagascar. Am J Trop Med Hyg. 2018;98:1683–91.
32. Wurtz N, Briolant S, Lemarié D, Pommier de Santi V, Pascual A, Roodt T, et al. Delayed diagnosis of *Plasmodium falciparum* in a soldier in Uganda: false-positive rapid diagnostic test associated with reduced repeats in pfhrp2. Med Sante Trop. 2013;23:181–4 (**in French**).
33. Kumar N, Singh JPN, Pande V, Mishra N, Srivastava B, Kapoor R, et al. Genetic variation in histidine rich proteins among Indian *Plasmodium falciparum* population: possible cause of variable sensitivity of malaria rapid diagnostic tests. Malar J. 2012;11:298.
34. Alemayehu GS, Messele A, Blackburn K, Lopez K, Lo E, Janies D, et al. Genetic variation of *Plasmodium falciparum* histidine-rich protein 2 and 3 in Assosa zone, Ethiopia: its impact on the performance of malaria rapid diagnostic tests. Malar J. 2021;20:394.
35. Dejazmach Z, Alemu G, Yimer M, Tegegne B, Getaneh A. Prevalence of malaria and associated knowledge, attitude, and practice among suspected patients in Bahir Dar Zuria district, Northwest Ethiopia. Biomed Res Int. 2021;2021:3741413.
36. Abossie A, Yohanes T, Nedu A, Tafesse W, Damitie M. Prevalence of malaria and associated risk factors among febrile children under five years: a cross-sectional study in Arba Minch Zuria District, South Ethiopia. Infect Drug Resist. 2020;13:363–72.
37. Massebo F, Balkew M, Gebre-Michael T, Lindtiørn B. Entomologic inoculation rates of *Anopheles arabiensis* in southwestern Ethiopia. Am J Trop Med Hyg. 2013;89:466–73.
38. Rosen M, Gatton L. Obare method calculator v.1.0. 2015. https://www.iddo.org/document/obare-method-calculator. Accessed 15 June 2022
39. Hofmann N, Mwingira F, Shekalaghe S, Robinson LJ, Mueller I, Felger I. Ultra- sensitive detection of *Plasmodium falciparum* by amplification of multi-copy subtelomeric targets. PLoS Med. 2015;12: e1001788.
40. WHO. Guidelines for malaria. Geneva: World Health Organization; 2022.
41. Costa GL, Mascarenhas ME, Martin TOG, Fortini LG, Louzada J, Pereira DB, et al. A comprehensive analysis of the genetic diversity of *Plasmodium falciparum* histidine-rich protein 2 (PfHRP2) in the Brazilian Amazon. Front Cell Infect Microbiol. 2021;11: 742681.
42. Nderu D, Kimani F, Thiong K, Karanja E, Akinyi M, Too E, et al. *Plasmodium falciparum* histidine-rich protein (PfHRP2 and 3) diversity in Western and Coastal Kenya. Sci Rep. 2019;9:1709.
43. Addai-Mensah O, Dinko B, Noagbe M, Ameke SL, Annani-Akollor ME, Owiredu EW, et al. *Plasmodium falciparum* histidine-rich protein 2 diversity in Ghana. Malar J. 2020;19:256.
44. Li P, Xing H, Zhao Z, Yang Z, Cao Y, Yan G, et al. Genetic diversity of *Plasmodium falciparum* histidine-rich protein 2 in the China Myanmar border area. Acta Trop. 2015;152:26–31.
45. Zeleke AJ, Hailu A, Bayih AG, Kefale M, Amare AT, Tegegne Y, et al. *Plasmodium falciparum* histidine-rich protein 2 and 3 genes deletion in global settings (2010–2021): a systematic review and meta-analysis. Malar J. 2022;21:26.
46. Agaba BB, Yeka A, Nsobya S, Arinaitwe W, Nankabirwa J, Opigo J, et al. Systematic review of the status of pfhrp2 and pfhrp3 gene deletion, approaches and methods used for its estimation and reporting in *Plasmodium falciparum* populations in Africa: review of published studies 2010–2019. Malar J. 2019;18:355.
47. Kosakovsky Pond S, Posada D, Gravenor M, Woelk C, Frost S. Automated phylogenetic detection of recombination using a genetic algorithm. Mol Biol Evol. 2006;23:1891–901.
48. Lee N, Gatton ML, Pelecanos A, Bubb M, Gonzalez I, Bell B, et al. Identification of optimal epitopes for *Plasmodium falciparum* rapid diagnostic tests that target histidine-rich proteins 2 and 3. J Clin Microbiol. 2012;50:1397–405.
49. Nyataya J, Waitumbi J, Mobegi VA, Noreddin A, El Zowalaty ME. *Plasmodium falciparum* histidine-rich protein 2 and 3 gene deletions and their implications in malaria control. Diseases (Basel). 2020;8:15.
50. Ramutton T, Hendriksen IC, Mwanga-Amumpaire J, Mtove G, Olaosebikan R, Tshefu AK, et al. Sequence variation does not confound the measurement of plasma PfHRP2 concentration in African children presenting with severe malaria. Malar J. 2012;11:276.

Publisher's Note