Deletion patterns, genetic variability and protein structure of pfhrp2 and pfhrp3: implications for malaria rapid diagnostic test in Amhara region, Ethiopia

Irene Molina - de la Fuente1,2,3*, Mulat Yimar4, Luz García2,5, Vicenta González2,5, Arancha Amor6, Melaku Anegagrie6, Agustín Benito2,5, Javier Martínez1, Marta Moreno7 and Pedro Berzosa2,5

Abstract

Background: Although rapid diagnostic tests (RDTs) play a key role in malaria-control strategies, their efficacy has been threatened by deletion and genetic variability of the genes pfhrp2/3. This study aims to characterize the deletion, genetic patterns and diversity of these genes and their implication for malaria RDT effectiveness, as well as their genetic evolution in the Amhara region of Ethiopia.

Methods: The study included 354 isolates from symptomatic patients from the Amhara region of Ethiopia who tested positive by microscopy. Exon 1–2 and exon 2 of genes pfhrp2 and -3 were amplified, and exon 2 was sequenced to analyse the genetic diversity, phylogenetic relationship and epitope availability.

Results: The deletion frequency in exon 1–2 and exon 2 was 22 and 4.6% for pfhrp2, and 68 and 18% for pfhrp3, respectively. Double deletion frequency for pfhrp2 and pfhrp3 was 1.4%. High genetic diversity, lack of clustering by phylogenetic analysis and evidence of positive selection suggested a diversifying selection for both genes. The amino-acid sequences, classified into different haplotypes, varied widely in terms of frequency of repeats, with novel amino-acid changes. Aminoacidic repetition type 2 and type 7 were the most frequent in all the sequences. The most frequent epitopes among protein sequences were those recognized by Mabs 3A4 and C1-13.

Conclusion: Deletions and high amino acidic variation in pfhrp2 and pfhrp3 suggest their possible impact on RDT use in the Amhara region, and the high genetic diversity of these genes could be associated with a diversifying selection in Ethiopia. Surveillance of these genes is, therefore, essential to ensure the effectiveness of public health interventions in this region.

Keywords: Malaria, Ethiopia, Plasmodium falciparum, pfhrp2, Rapid diagnostic test, Deletions
Background

Malaria, the cause of 627,000 deaths in 2020, is concentrated in Africa, with the majority of cases being caused by *Plasmodium falciparum* [1]. According to World Health Organization (WHO), malaria-control programmes are expected to rely on test-and-treat strategies, where the effectiveness of diagnosis is essential to ensure a prompt treatment [2]. In this regard, rapid diagnostic tests (RDTs) are becoming increasingly popular as they provide a simple and cheap diagnosis [3].

RDTs are based on detection of specific parasitic proteins [4], and the majority of RDTs for *P. falciparum* rely on the detection of *P. falciparum* histidine rich protein-2 (PfHRP2), which reports cross-reactions with *P. falciparum* histidine rich protein-3 (PfHRP3) [5, 6]. PfHRP2 is the preferred protein for RDTs due to its stability, specificity and abundant expression during the blood stage [7].

The *pfhrp2* and *pfhrp3* genes comprise two exons, namely exon 1–2, which contains signal and cleavage sequences, and exon 2, which is the encoding sequence [8]. Deletions in *pfhrp2* and *pfhrp3* could be the cause of misdiagnosis and of false-negative *P. falciparum* results in HRP2-based RDTs [9, 10]. This could have an impact on delay or lack of treatment, with consequences for both the patient and for the maintenance of malaria transmission [11, 12]. In addition, *pfhrp2* and *pfhrp3* are highly variable genes with tandem repetitions [13, 14].

Variations in amino acid repeat motifs have also been reported to be a factor that may modify the effectiveness of HRP2-based RDTs [15, 16]. In this regard, although the main epitopes of PfHRP2 have been identified previously [17], the protein structures of PfHRP2 and PfHRP3 are still unknown, which complicates prediction of the epitope availability or the effect of polymorphisms [18, 19].

The dynamics of the evolution of these genes is not well understood. Thus, although there is evidence of selective pressure on *pfhrp2* and *pfhrp3* [20, 21], including in Ethiopia [22], multiple origins of parasites with deletions have also been reported suggesting spontaneous and multiple deletion events [23, 24].

Ethiopia is the most populous country in eastern Africa and reported approximately 260,000 malaria cases in 2019, with 5626 deaths [25]. However, it is considered to be a country in the process of consolidating malaria control, with this disease being heterogeneously distributed [26]. Amhara has a low-moderate malaria transmission, with a *Plasmodium sp.* prevalence of 0.8% in 2015 [27].

HRP2-based RDTs were implemented in Ethiopia in 2004 and have been used since 2010 as evidence for treatment as part of a test-and-treat policy [28]. Since
then, however, deletions in \textit{pfhrp2} and \textit{pfhrp3} genes have been reported in some regions of Ethiopia [22, 29, 30], and neighbouring countries, such as Kenya [31] and Eritrea [10], thereby jeopardizing the efficacy of test-and-treat policies and efforts made by National Malaria Programmes in their implementation. As such, an increased understanding of the status of these genes and their genomic dynamics could help us to better understand their dynamics and design future guidelines. Moreover, there are no studies in Ethiopia focussing on the structural diversity of HRP2 and HRP3 proteins.

In light of the above, the aims of this study were to characterize the deletion prevalence and patterns of exons 1–2 and 2 of the \textit{pfhrp2} and \textit{pfhrp3} genes in the Amhara region and to study their genetic diversity, using the parameters diversity and haplotype networks, along with the amino-acid sequence variations in exon 2. The predicted sensitivity of HRP2-based RDT was also assessed using a theoretical model and the frequency and localization of epitopes on predicted protein structure models. Also assessed was the possible force driving selection and the evolution of these genes by performing a phylogenetic and genetic evolution analysis.

\section*{Methods}

\subsection*{Sample collection and study area}

The study was conducted in the Amhara region, Bahir Dar Department, Ethiopia (East Africa). During the malaria transmission season in 2014 (September to December), samples from symptomatic malaria infections from outpatients at health facilities were collected. Febrile or otherwise malaria-symptomatic patients aged \(\geq 16\) years with positive \textit{P. falciparum} diagnosis by microscopy were eligible to participate. Samples were collected at four health facilities: Bahir Dar city, the principal city of the region near to Tana Lake (population: 170,000); Tis Abay, a town surrounded by farming areas close to the Blue Nile Falls waterfall; Meshenti, a medium-sized city to the southwest of Bahir Dar city; and, Zenzelima, a rural and surrounding area to the northeast of Bahir Dar city (Fig. 1). This region has a sub-tropical highland oceanic climate, with temperatures ranging between 18 and 24 °C (an average temperature of 20 °C), and abundant precipitation concentrated in the rainy season (rainfall around 1850 mm per year).

Blood samples were taken on Whatman 903TM paper (GE Healthcare Bio-Sciences Corp.) by finger prick and stored in double zip-lock plastic bags with silica gel at \(-20\) °C, and while they were being analysed they were stored at 4 °C. They were then transported to the National Centre for Tropical Medicine, Institute of Health Carlos III (Madrid, Spain) for molecular and genetic analysis.

\subsection*{DNA extraction and molecular diagnosis of \textit{Plasmodium sp.}}

DNA was extracted from dot blood spots on filter paper using the chelex–saponin method [32]. A 5-mm diameter
punch containing 10 μL of blood was used [33]. Malaria molecular diagnosis was performed on all samples using nested multiplex PCR targeting the 18S small sub-unit ribosomal RNA (ssRNA) to confirm *P. falciparum* infections [34, 35]. Amplification of other *P. falciparum* genes (*pfdhfr, pdhps, pfmdr1, pfcr*) was performed to ensure that the samples were free of PCR-inhibiting elements and thus confirm that when the *pfhrp2* and *pfhrp3* genes did not amplify it was due to a true deletion and not inhibition.

**pfhrp2 and pfhrp3 gene deletions**

Amplification of exon 2 of *pfhrp2* and *pfhrp3* was performed by semi-nested PCR following a previously described procedure [10] and amplification of exon 1–2 of *pfhrp2* and *pfhrp3* was performed following a protocol with slight modifications [36], such as a lower annealing temperature (55 °C) and more cycles for both PCRs (35 cycles). A summary of the workflow is described in Fig. 2. PCR amplicons were separated and visualized on a 2% agarose gel stained with Pronasafe (Pronadisa SA, Spain). If no amplification was

---

**Fig. 2** Methodological flow scheme. Microscopy diagnosis was confirmed by nested multiplex PCR, distinguishing *P. falciparum*, *Plasmodium vivax*, *Plasmodium Ovale*, and *Plasmodium malaria*. Four independent PCRs were run for the *P. falciparum* samples to detect deletions in exon 1–2 and exon 2 of *pfhrp2* and *pfhrp3*. The deletion of any exon was confirmed by an absence of amplification after three PCR repetitions and the confirmation of maintained DNA quality. Samples that could not be confirmed for DNA quality were excluded from the analysis for that gene. Subsequent data analysis for the combined results was performed only with those samples included in the independent analysis for both genes. Additionally, a sub-sample lacking the deletion for exon 2 of both genes was sequenced and the genetic diversity and variation in amino-acid sequences analysed. Finally, a sub-sample of these sequences was used to predict the protein structure model and to locate the epitopes in the structure.
observed, the PCR experiment was repeated, and, only for exon 2 of both genes, if the result was still negative, it was repeated a third time with a lower elongation temperature (60 °C) [37]. If there was no amplification in any of the experiments, the maintenance of DNA quality was confirmed by nested multiplex PCR, and if amplification was observed, the result was considered to be a deletion event.

Samples with deletion in exon 1–2 but not in exon 2, or vice versa, were also amplified using the forward primer for exon 1–2 and the reverse primer for exon 2, and the previously reported PCR protocols for exon 2 of both genes. Samples that did not amplify with that PCR were assumed to have had some part of the exon that did not amplify also not previously deleted. *Plasmodium falciparum* clone 3D7 was used as a positive control in all PCRs, with clone Dd2 as negative control for the *pfhrp2* gene amplification. This clone contains a *pfhrp2* gene deletion. Similarly, clone HB3, with *pfhrp3* deleted, was used as negative control for the *pfhrp3* gene amplification.

**Genetic diversity of exon 2 from *pfhrp2* and *pfhrp3***

**Sequencing and genetic analysis of exon 2 from *pfhrp2* and *pfhrp3***

A random sub-sample of PCR-positive samples for both genes (*pfhrp2* and *pfhrp3*) was sequenced. The PCR products were purified using Illusta Exoprostar 1-step (GE Healthcare Life Sciences) following the manufacturer’s instructions. Samples were sequenced in both directions using forward and reverse primers for the second PCR of exon 2 at a concentration of 6 pmol/μL, sequencing using a standard dye terminator in an ABI PRISM 3730 XL Analyser (Big Dye Terminator v3.1 Cycle Sequencing kit).

Bio Edit Sequence Alignment Editor Software v7.1.3.0 was used for sequence editing and solving undetermined sites. Mega7 v7.0.26 was used for multi-sequence alignment with ClustalW tool, a progressive alignment method recommended for data sets with varying degrees of divergence [38]. Polymorphism was then analysed by determining the diversity of haplotypes, nucleotide diversity (π) and genetic diversity (Θ) using DNAsp v6.12.03. Diversity was analysed through pairwise comparison, where gaps/missing presented in each comparison were excluded but only for that comparison. Haplotype networks were constructed using the Median Joining Network method, which is phylogeographical analysis with lack of rooting, in PopART v.1.7. The randomness of the distribution of haplotypes between populations was assessed using the Exact test for population differentiation based on Haplotype Frequencies calculated in Arlequin v3.5.2.2. Neutrality tests, namely Tajima’s D, which is based on allele frequencies, and Fu and Li’s D*, which analyses synonymous and non-synonymous sites, were calculated using DNAsp v6.12.03 as a means of detecting selection.

**Phylogenetic analysis**

The evolutionary relationships of exon 2 *pfhrp2* and *pfhrp3* were assessed for the same sub-sample of nucleotide sequences sequenced for genetic analysis using the Datamonkey server [39]. First, genetic recombination was evaluated using the Genetic Algorithm for Recombination Detection (GARD) tool [40]. GARD is a pre-processing step for selection inference because it groups unique phylogenetic histories for each recombination block. The Branch-site Unrestricted Statistical Test for Episodic Diversification (BUSTED) tool was then applied to detect gene-wide positive selection at least one site on at least one branch [41].

To assess phylogenetic relationships combined with clustering by geographical location. Phylogenetic trees were performed using Bayesian Inference by MrBayes v3.2.7a and Maximum Likelihood by Mega7 v7.0.26 as inference methods to estimate evolutionary distances between all sequences. Both methods used substitution models GTR+T+I, where missing data or alignments gaps were not considered, so loci with gaps or missing data were eliminated of the analysis. In this analysis, 33 homologous sequences of each gene from countries bordering Ethiopia and other regions of Sub-Saharan Africa were included, published in GenBank (Additional file 1).

**Study of amino acid sequences and protein structure**

**Variation in amino acid repeats patterns**

All the sequenced samples were used to amino acid repeats characterization with a number according to Baker et al. [15] and recent repeats characterized in Kenya [42]. When two or more amino acid repeats overlapped, only the largest repeat was counted. Analysis of the frequency and organization of amino acid repeats was carried out with a custom code using Python 3.9 Software (Additional file 2).

**In silico translation of exon 2 of *pfhrp2* and *pfhrp3***

Prediction of the secondary and tertiary structure of the protein was performed using two different platforms to obtain more consistency in the results: (1) RaptorX (http://raptورx.uchicago.edu); and (2) Protein Homology/analogy Recognition Engine v2.0 (PHYRE2) (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). Both analyses applied a hierarchical approach with a combination of derived-template and de novo protein prediction to generate full-chain models. Firstly, amino
acidic sequences were submitted in PHYRE2 for a batch-processing, then sequences with higher quality and confidence were selected for following analysis, structure prediction by RaptorX. RaptorX showed the best results for secondary and tertiary structures. Model quality was estimated using the RMSD (root mean square deviation) value and the model with lowest RMSD was selected as this indicates the deviation observed between the native and predicted positions [43]. Additionally, PHYRE2 was used for structure-based function prediction, according to the alignments.

Description and localization of epitopes in the protein

The presence of 11 linear epitopes, previously described as the epitopes for optimal monoclonal antibodies potentially used in HRP2-based RDTs, as information about mAbs used in commercial RDT kits is unavailable, was confirmed in protein sequences and the frequency of each epitope per sequence was analysed. The median repetition of each epitope, and its frequency among the sequences, was calculated. The PyMOL2 molecular graphic system v.2.5 (https://pymol.org/2/) was used to locate the epitopes in the predicted tertiary structure and APBS [44] and PDB2PQR [45] were used to calculate the Solvent Accessible Surface area.

Results

Deletions in pfhrp2 and pfhrp3 genes

A total of 354 filter paper samples were collected from symptomatic patients with malaria (diagnosed by microscopy) from Tis Abay (N = 200), Bahir Dar (N = 81), Zenzelima (N = 35), and Meshenti (N = 38). Of these, 52 samples were excluded due to low DNA quality. Plasmodium falciparum was confirmed in most of the samples (294/302; 97.35%), P. vivax was identified in 2.65% (8/302) and no mixed infections were detected. Plasmodium falciparum samples were used for amplification of exon 1–2 and exon 2 of pfhrp2 and pfhrp3 genes. After the confirmation of deleted samples (i.e., deletion in pfhrp2 and/or pfhrp3 genes) 280 samples were included for the pfhrp2 analyses and 238 samples for the pfhrp3 analyses (Fig. 3). Negative samples that could not be confirmed to maintain sufficient DNA quality were excluded from the analysis (n = 5 for pfhrp2 analysis only, n = 47 for pfhrp3 analysis only, and n = 9 for both pfhrp2 and pfhrp3 analysis). For joint outcomes of both genes, 219 samples were included as they were only samples that were not excluded from any of the analyses.

Frequency of deletion in exons 1–2 and 2 of pfhrp2 and pfhrp3 genes

Deletions were detected in exons 1–2 and 2 for both genes, with a higher frequency of deletions in exon 1–2
(19.29% (54/280) for pfhrp2 and 68.49% (163/238) for pfhrp3) than in exon 2 (4.64% (13/280) for pfhrp2 and 18.49% (44/238) for pfhrp3). Candidate partial deletion was considered when only exon 1–2 or 2 have amplified but ‘partial deletions’ could not be confirmed. Full-length PCR was performed for partially deleted candidate samples, but only 5 of 51 of pfhrp2 and 14 of 123 pfhrp3 amplified with that PCR showing different fragment sizes than expected (Fig. 4).

![Gel images of the full-length PCR of pfhrp2 and pfhrp3. Electrophoresis gel showed the result of the amplification for PCR combining exon 1–2 and exon 2, the expected fragment was around 900 base pairs as it could be observed in the positives controls placed at the end of the gel: 3D7, Dd2 and HB3. Negative controls for the first and second PCR were placed in the first two wells of the gel.](image)

**Table 1** Deletion patterns for exon 1–2 and exon 2 of pfhrp2 and pfhrp3 genes

|          | Exon 1–2 | Exon 2 | F (90% IC)     | n (N)  |
|----------|----------|--------|----------------|--------|
| *pfhrp 2*|          |        |                |        |
| Complete deletion       | –        | –      | 2.86 (1.49–5.22) | 8 (280) |
| Candidate partial deletion | –     | +      | 16.43 (12.94–20.58) | 46 (280) |
| Candidate partial deletion | +     | –      | 1.79 (0.76–3.86)  | 5 (280)  |
| Complete gene           | +        | +      | 78.93 (74.46–82.82) | 221 (280) |
| *pfhrp 3*|          |        |                |        |
| Complete deletion       | –        | –      | 17.64 (13.76–22.29) | 42 (238) |
| Candidate partial deletion | –     | +      | 50.84 (45.32–56.34) | 121 (238) |
| Candidate partial deletion | +     | –      | 0.84 (0.18–2.81)  | 2 (238)  |
| Complete gene           | +        | +      | 30.67 (25.16–36.80) | 73 (238) |

Exon 1–2: samples that did not amplify for exon 1–2; Exon 1–2 +: samples that amplified for exon 1–2; Exon 2: samples that did not amplify for exon 2; Exon 2 +: samples that amplified for exon 2.
Pfhrp2 presented at least one deleted exon in 21.08% of isolates and 2.86% of them had the pfhrp2 gene completely deleted (any exon amplified) (Table 1). A high proportion of isolates presented some deletion in pfhrp3 (73.95%), with complete deletion of this gene being detected in 18.49% of isolates. There were significant differences in the frequency of exon 2 of pfhrp3 deletion by location (p-value < 0.05), but not for exon 2 of pfhrp2. Frequencies of exon 2 of pfhrp2 deletion are similar between locations but pfhrp3 deletion showed significant differences by location (Additional file 3).

**Deletion patterns in pfhrp2 and pfhrp3**

Overall, double-negative samples (pfhrp2 and pfhrp3 completely deleted) were found in 1.37% (3/219) of cases and both complete genes were identified in 30.59% (67/219) (Table 2).

| Pfhrp2 | Pfhrp3 | F (90% IC) | Number of isolates (N = 219) |
|--------|--------|------------|-------------------------------|
| Exon 1–2 | Exon 2 | Exon 1–2 | Exon 2 |
| + | + | + | + | 30.59 (25.52–36.16) | 67 |
| – | – | + | + | 0 | 0 |
| + | + | – | – | 13.70 (10.12–18.22) | 30 |
| – | + | – | + | 8.68 (5.84–12.59) | 19 |
| + | – | + | – | 0.46 (0.01–2.38) | 1 |
| – | – | – | – | 1.37 (0.42–3.69) | 3 |

**Genetic diversity of exon 2 of pfhrp2 and pfhrp3 genes**

A total of 95 pfhrp2 and 79 pfhrp3 exon 2 P. falciparum sequences were analysed and translated successfully into amino acids (aa). These sequences were submitted to the GenBank database and the homology with the pfhrp2 and pfhrp3 genes of P. falciparum checked for further analysis. The size of the exon 2 sequence ranged from 400 to 750 bp in pfhrp2 and 350 to 700 bp in pfhrp3, with an average of 210 and 188 amino acids, respectively. A total of 92 haplotypes were identified in the pfhrp2 sequences, with 93.68% of sequences being unique (89/95). All the haplotypes shared by more than one sample include only two sequences (Additional file 4). A total of 77 haplotypes were detected for the pfhrp3 sequences, with two haplotypes shared by two samples from different health centre. All haplotype sequences were submitted to the GenBank database (accession numbers: OL961136–OL961227 and OL961228–OL961304).

The haplotype analysis of pfhrp2 showed a higher diversity, in terms of haplotype distribution and frequency, than the haplotype network for pfhrp3, for which lower distribution for the different haplotypes were observed (Additional file 4). These results are consistent with the genetic diversity analysis, which revealed a higher nucleotide diversity for pfhrp2 ($\pi = 0.158$) than for pfhrp3 ($\pi = 0.027$) (Table 3).

Test statistics for neutrality were applied to assess the hypothetical evolution of the genes. The neutrality tests for pfhrp2 were both negative (Fu and Li’s $D^* = -2.43$ and Tajima’s Neutrality Test $D = -0.96$), but only Fu and Li’s $D^*$ was statistically significant (p-value < 0.05). In contrast, the neutrality tests for pfhrp3 were both negative (Tajima’s $D = -2.23$ and Fu and Li’s $D^*$

| Health centre | No. of isolates | S | H | K | $\pi$ | $\Theta$ per site | $\Theta$ per sequence |
|---------------|----------------|---|---|---|------|------------------|----------------------|
| Pfhrp2 gene   |                |   |   |   |      |                  |                      |
| Overall       | 95             | 532 | 92 | 63.36 | 0.158 | 0.162 | 135.53 |
| Tis Abay      | 57             | 419 | 55 | 61.32 | 0.150 | 0.147 | 108.07 |
| Bahir Dar     | 16             | 305 | 16 | 65.13 | 0.175 | 0.180 | 108.73 |
| Zenzelima     | 3              | 532 | 3  | 63.36 | 0.159 | 0.162 | 135.54 |
| Meshenti      | 19             | 320 | 19 | 65.46 | 0.163 | 0.151 | 114.65 |
| Pfhrp3 gene   |                |   |   |   |      |                  |                      |
| Overall       | 79             | 188 | 77 | 10.223 | 0.027 | 0.083 | 49.02 |
| Tis Abay      | 50             | 140 | 48 | 9.01  | 0.025 | 0.071 | 41.75 |
| Bahir Dar     | 13             | 91  | 13 | 13.41 | 0.035 | 0.068 | 34.29 |
| Meshenti      | 16             | 57  | 16 | 10.11 | 0.023 | 0.049 | 25.06 |

Results were obtained using pairwise comparison, 4,465 pairwise comparisons have been performed and the average number of sites analysed by comparison was 405.72, total number of sites were 916.

S: number of segregating sites; H: number of different haplotypes; K: average number of nucleotide differences; $\pi$: nucleotide diversity; $\Theta$ per site: genetic diversity per site; $\Theta$ per sequence: genetic diversity per sequence.
test statistic = −6.05) and statistically significant (p-value < 0.05).

**Phylogenetic and evolution analysis**

The ClustalW alignment resulted in an alignment of 916 base pairs for pfhrp2 and 618 sites for pfhrp3.

Potential recombination breakpoints between the pfhrp2 and pfhrp3 sequences were detected by GARD analysis and were considered for a subsequent evolution analysis. BUSTED analysis found evidence for gene-wide episodic diversifying selection at at least one site on at least one branch for both pfhrp2 and pfhrp3, with a synonymous rate variation (ω) > 1. These results were confirmed by repeating the same analysis including homologous sequences from other regions of sub-Saharan Africa from GenBank.

The Bayesian inference phylogenetic tree showed an unclear clustering by health centre, country or region for both genes (Fig. 5). The Maximum Likelihood Inference tree showed similar results. Sequences from the same health centre tend to form small clusters but without being truly isolated. Similarly, sequences from countries bordering Ethiopia (Kenya and Sudan) were more closely related to sequences from the present study. However, sequences from other African regions are also relatively closely related to some sequences, thus indicating an unclear geo-clustering.

**Variation in amino-acid repeat patterns**

Differences in the frequency and organization of amino-acid repeats were detected, with the most frequent amino acids in pfhrp2 and pfhrp3 being alanine (37.32%), histidine (36.16%) and aspartate (9.59%), and histidine (31.18%), alanine (30.19%), asparagine (9.55%), and aspartate (9.11%), respectively.

**Variation in amino-acid repeats in pfhrp2**

A total of 20 previously reported amino-acid repeat types were found, with repeat types 2 and 7 being observed in all isolates and with the highest average repetition frequency (type 2: 8.8-times and type 7: 4.3-times) (Table 4). The next most frequent repeats were type 3 (83%) and type 8 (90%), which appeared in the range 0–2-times per sequence, and type 4 (84%), for which the repetition times per sequence ranged from 0- to 18-times. New or modified repeat amino-acid motifs were detected and classified (Additional file 5). Organization of the

![Fig. 5 Bayesian inference phylogenetic tree for pfhrp2 (a) and pfhrp3 (b) sequences.](image)
amino-acid repeats in the sequences followed similar patterns, although with some variations (Additional file 6).

**Variation in amino-acid repeats in pfhrp3**
A total of 15 previously reported amino-acid repeat types were found in the sequences (Table 4). Repeat type 16 was included in the majority of isolates (99%) and was the most repeated type in the sequences (average time: 11.15). The following more frequent types had markedly fewer repeats per sequence. For example, types 7 and 20 were found in 95% and 88% of samples, respectively, but only one time per sequence. The organization of amino-acid repeats was similar for all samples and in agreement with previously reported results (Additional file 7).

**Table 4 Occurrence and number of amino-acid repeat types in pfhrp2 and pfhrp3 sequences**

| Repeat types | Amino acid repeat | Pfhrp2 Range of no. of repeats | Occurrence (%) | Pfhrp3 Range of no. of repeats | Occurrence (%) |
|--------------|-------------------|--------------------------------|----------------|--------------------------------|----------------|
| Type 1       | AHHAHHAVD         | 0–5                            | 42             | 0–2                            | 63.75          |
| Type 2       | AHHAPHAD          | 1–16                           | 100            | 0                              | 0              |
| Type 3       | AHHAHHAAY         | 0–2                            | 83             | 0                              | 0              |
| Type 4       | AHH              | 0–18                           | 80             | 0–6                            | 88.75          |
| Type 5       | AHHAHASD          | 0–2                            | 67             | 0                              | 0              |
| Type 6       | AHHTD             | 0–7                            | 75             | 0                              | 0              |
| Type 7       | AHHAAD            | 1–8                            | 100            | 0–1                            | 95.00          |
| Type 8       | AHHAAY            | 0–2                            | 90             | 0                              | 0              |
| Type 9       | AAY               | 0–1                            | 3              | 0                              | 0              |
| Type 10      | AHHAAAHHTT       | 0–3                            | 40             | 0                              | 0              |
| Type 11      | AHN               | 0                              | 0              | 0                              | 0              |
| Type 12      | AHHAAHHEATH      | 0–1                            | 35             | 0                              | 0              |
| Type 13      | AHHASD            | 0–2                            | 18             | 0                              | 0              |
| Type 14      | AHHHAAD           | 0–1                            | 13             | 0                              | 0              |
| Type 15      | AHHHAAN           | 0                              | 0              | 0–1                            | 68.75          |
| Type 16      | AHHAAAN           | 0–1                            | 1               | 0–17                           | 98.75          |
| Type 17      | AHHDG             | 0                              | 0              | 0–8                            | 90.00          |
| Type 18      | AHDD              | 0                              | 0              | 0–4                            | 88.75          |
| Type 19      | AHHAA             | 0–2                            | 23              | 0–1                            | 1.25           |
| Type 20      | SHHDD             | 0                              | 0              | 0–1                            | 87.5           |
| Type 21      | AHHAHATY          | 0                              | 0              | 0                              | 0              |
| Type 22      | AHHAHAGD          | 0                              | 0              | 0                              | 0              |
| Type 23      | ARHAAD            | 0                              | 0              | 0                              | 0              |
| Type 24      | AHHHTHAAD         | 0                              | 0              | 0                              | 0              |

...continued...

**Structure prediction and epitope localization**

*In silico structure prediction*
The secondary and tertiary protein structure was predicted for a sub-set of samples (n = 22), selected based on sequence quality, using RaptorX. For most samples, the majority of the secondary structure was a helix, with some coils placed principally in intermediate positions. Batch Phyre2 structure-based function prediction analyses were carried out for 96 samples, with a significant result being found for only 10 samples: significant results are needed for structure prediction and homology. Among these, the main structure matches were with proteins with a hydrolase function (Additional file 8).

*Epitopes of HRP2 and HRP3 targeted by monoclonal antibodies*
Seven of the 11 major antibody epitopes were identified in more than 84% of pfhrp2 sequences (n = 81) (Table 5). Two epitopes, namely 3A4 and C1-13, were present in all sequences with the highest median frequencies: 14- and 13-times per sequence, respectively. Epitopes 2G12-1C12, N7 and PTL-3 were present in 99, 99 and 95% of sequences, respectively, but at lower frequencies (2–4-times per sequence).

Among pfhrp3 sequences, epitope 3A4 was the most common (70%), but with only a low frequency (median = 1), followed by 1E1-A9, which was found in 65% of sequences (median = 1).
The eight tertiary structure models were used to locate the main epitopes in the structure. These were then compared with the solvent-accessible surface area, which has a mainly negative electrostatic potential. Epitopes 3A4 and C1-13 were found to be the most accessible as they were widely distributed over the protein surface, whereas other epitopes were located in only a specific region of the surface (Fig. 6). Additionally, epitopes recognized by 3A4 were also found in the PfHRP3 models studied, but with less accessibility (Fig. 7).

**Discussion**

An understanding of *pfhrp2* and *pfhrp3* gene deletions and genetic diversity is key to the design of future interventions in RDT-based malaria test-and-treat strategies. This study describes the frequency of deletions and unique data regarding the sequence variation of *pfhrp2* and *pfhrp3* from the Amhara region in Ethiopia.

**pfhrp2 and pfhrp3 deletions**

This study showed that the detected frequency of the exon-2 deletion for *pfhrp2* in the Amhara region of Ethiopia (4.64%) is worryingly close to the threshold (5% at the lower end of the 90% CI for false negatives by HRP2-based RDT) established by the WHO to change the use of HRP2-based RDTs [46]. Although this study did not use results from RDT, a higher prevalence of deletion among HRP2-based RDT false-negatives than for samples diagnosed by microscopy, where false negatives and true positives by HRP2-based RDT could be mixed, is to be expected. Taking this into account, the higher frequency of deletion (11.5%) reported for RDT false-negatives in 2018 in Amhara suggests a gradual increase [22]. In contrast, a higher frequency of deletion has been reported in other regions of Ethiopia, for example 18% among samples including true positives and false negatives and 42% among HRP2-based RDT false-negatives in the Assosa region, with high/moderate transmission [29], and 100% among general samples in Oromia, with low transmission [30]. The differences between reported prevalence could be related to the association between deletion frequency and low malaria transmission, where it is less probable to find a mixed infection of parasites with and without *pfhrp2* that masks the deletion [20]. Another explanation for the wide range of deletion prevalence reported could be the differences in genotyping approach, for example, a recent study in Ethiopia only analysed samples with low or without HRP2 signal by multiplex bead assay [47]. That makes it essential to generalized technical strategies across studies [48]. Potential false negatives caused by *pfhrp2* deletions could be avoided by cross-reaction with PfHRP3 as this can be detected by HRP2-based RDT in the absence of pfHRP2 [4]. However, in the light of this study’s results, in Ethiopia this is unlikely to be possible due to the high frequency of *pfhrp3* deletions (18.5%) and isolates with deletions in both genes.

With regard to patterns of deletions, the findings are in agreement with the literature, which shows that deletions in exon 1–2 and in *pfhrp3* are more common than in exon 2 and in *pfhrp2*, respectively [49, 50]. This may suggest the possibility of sequential deletion, starting with deletion in exon 1–2 but ending with complete deletion [51]. But, deletions in only one exon, called in this manuscript ‘candidate partial deletion’, could not be confirmed and could be simply due to worse amplification in exon 1–2. However, deletions in different fragments of these genes have also been reported, suggesting that the full gene is not always deleted [22]. Evolution and selection

**Table 5** Frequency of major epitopes in *pfhrp2* and *pfhrp3* exon-2 targeted by MAbs in HRP2-based RDT

| MAb   | Major epitope | Pfhrp2 Frequency (%) | Median frequency (n) | Pfhrp3 Frequency (%) | Median frequency (n) |
|-------|---------------|----------------------|----------------------|----------------------|----------------------|
| 3A4   | AHHAHHA       | 100                  | 14                   | 70                   | 1                    |
| 2G12-1C12 | DAHHAAADAHH   | 99.06                | 4                    | 90                   | 0                    |
| 1E1-A9 | AHHAHV        | 42.06                | 0                    | 65                   | 1                    |
| A6-4  | HATDAHH       | 84.11                | 3                    | 0                    | 0                    |
| C1-13 | AHHHAADAHH    | 100                  | 13                   | 4                    | 0                    |
| N7    | DAHHAAADAHHA  | 99.06                | 4                    | 0                    | 0                    |
| PTL-3 | YAHHAHHA      | 95.33                | 2                    | 0                    | 0                    |
| S2-5  | AHHHASDAHHA   | 84.11                | 1                    | 0                    | 0                    |
| TC-10 | TDAAAAADAAADA  | 54.21                | 1                    | 0                    | 0                    |
| C2-3  | HAHHAADAHHA   | 43.93                | 0                    | 0                    | 0                    |
| Genway| AYAHHAHAAY     | 0                    | 0                    | 0                    | 0                    |
of *pfhrp* gene deletions will be closely associated with their fitness cost [52]. Although this remains to be clarified, previous studies in vitro have suggested the selection of strains without deletions and slower growth in strains with deletions [21, 53]. Moreover, in Ethiopia the extended use of combination HRP2/Pv or Pan—LDH-based RDT [54], due to high prevalence of *P. vivax*, could weaken the selection pressure of *pfhrp*-deleted parasites, unlike in other sub-Saharan countries.

**Genetic diversity and evolution analysis of exon 2 from *pfhrp2* and *pfhrp3* genes**

The dynamics of the spread or selection of *pfhrp2*-deleted populations remains unclear. However, as HRP2-based RDT use has increased exponentially in recent years, a selective pressure for parasites with *pfhrp2* deletions could be expected [31]. Indeed, linkage-specific clonal expansion of populations with *pfhrp2* deletions has been reported [55]. In contrast, the results of the BUSTED test showed diversifying expansion in the present study. This diversity, combined with a lack of clear clustering, could be related to an increased diversity step, when gene deletions occur, followed by clonal expansion promoted by selective pressure. In fact, recent strong selection of *pfhrp2*-deleted parasites have been reported from Ethiopia in a study carried out some years later, when this clonal expansion, boosted by test-track-treat policies, could be expected to happen [22]. Multiple spontaneous origins of deletions in these genes, which could be part of the diversifying expansion, have been reported previously [22, 56].

**Variation in amino-acid repeat patterns and its implication for the effectiveness of HRP2-based RDT**

The role of a variation in amino-acid repeats in RDT is unclear. Previous studies have reported both an influence on RDT results, increasing its sensitivity or causing misdiagnosis [57, 58], or a lack of association [59]. Parasites...
could maintain the gene but undergo changes in some specific amino-acid repeats, thereby becoming undetectable [60, 61]. Some repeat types could have more influence in HRP2-based RDT results, but there is not found a clear association [13]. This suggests the need to monitor the effectiveness of RDT.

The *pfhrp2* sequences showed 16 previously described repeat types, including some variations. The recent increase in reported variations and new domains is consistent with a diversifying expansion [40, 62]. In line with other studies, the most common repeats were types 2 and 7, the frequency of which markedly influences RDT detection [15, 63].

*Pfhrp3* sequences showed less diversity in terms of amino-acid sequences than *pfhrp2*, as reported previously [60]. Similar to previous studies, the most frequent and repeated type was type 16 [64, 65], and type 17 showed a marked variation, as in Kenya [61], thus suggesting a high antigenicity and that it may be useful for RDT detection.

**Protein structure prediction and epitope localization**

The relative similarity between the occurrence and organization of amino-acid repeats, either with geographically close parasites or those from other continents, contrasts with the high diversity detected at a nucleotide level. However, this could imply the temporal persistence of PfHRP2 epitopes despite the genetic diversity [61]. For instance, the results from the Amhara region are similar to those reported previously in other countries for the 12 available PfHRP2-specific MAbs. Although information about the antibodies used in commercial RDTs is unavailable, it is reasonable to assume that many of these epitopes are targeted [17]. The epitopes recognized by MAbs 3A4 and C1-13 were the most frequent [62, 66], and the epitope for 3A4 was also the most common in *pfhrp3* sequences [24]. Additionally, the epitope recognized by C1-13 was identified for the first time in the *pfhrp3* sequence in this paper. This detection of the usual epitopes increases the probability of parasite detection, even though they were present at only low frequencies.
Protein-structure models showed broad surfaces with solvent accessible surface areas on which the majority of linear epitopes were found and shown to be topographically available. In contrast, few of the linear epitopes were detected among HRP3 protein sequences, thus suggesting that, in this protein, the epitopes recognized by HRP2-RDT could be conformational rather than linear. Pfhhrp2 and pFhrp3 deletions might have a different impact depending on malaria endemicity. Low malaria diversity settings, which are related to a low prevalence of malaria, could lead to an easier selection of deleted parasites [67]. As both the competition and recombination between different strains of parasites are lower, deletion will be easier to maintain in following generations. The exception to this is parasites with both anti-malarial resistance and deletions, as they could be more easily selected irrespective of the conditions [20]. As such, surveillance of pFhrp2 and pFhrp3 is particularly important in settings in which malaria is decreasing as a result of prevention and control efforts, such as Ethiopia [68, 69]. Although HRP2-based RDTs are still considered useful for malaria diagnosis in endemic regions [70], WHO guidance recommends countrywide change when the threshold was exceeded in any region. Meanwhile, combination of pLDH-RDTs with targets other than HRP2 are preferable [9, 71].

There are some limitations to this study. For example, samples were collected based on microscopy smears positive for P. falciparum but RDT results or parasite densities were not available, thus limiting the power to detect the effect of deletions in malaria diagnosis. Furthermore, representativeness and comparisons between the different health centres was not possible epidemiologically due to the low number of samples. Also, candidate partial deletions could not be confirmed by genetic analysis. Moreover, genetic analysis of repetitive genes required the exclusion of large number of sites affecting genetic diversity results. However, the study still provides a more in-depth genetic characterization of pFhrp2 and pFhrp3 genes and their amino-acid composition. Subsequent studies should assess the implications of variations in sequences or epitopes and their effect on RDT results. Also, full-length of ‘partially deleted’ samples will provide insightful information about this partial deletions.

Conclusion
Pfhhrp2 and pFhrp3 deletions have been identified in P. falciparum isolates from Amhara region in Ethiopia. The high frequency of exon 2 pFhrp2 deletion and the detection of isolates with double deletions in both exon 2 of pFhrp2 and pFhrp3 could jeopardize malaria-control strategies based on diagnosis with HRP2-based RDTs.

The high genetic diversity and high polymorphism of the amino-acid sequences found in pFhrp2 and pFhrp3 may have an impact on RDT results and malaria diagnosis, especially given the relatively low frequency of repetition types 2 and 7 detected.

This study also provides insights into the localization of epitopes in the protein structure of pFhrp2 and pFhrp3, with the use of combinations of MAbs 3A4 and C1-13 being recommended to increase the reactivity of HRP2-based RDTs. However, a better understanding of protein structure could be useful for the design and evaluation of monoclonal antibodies for RDT.

In light of all these results and the malaria situation in Ethiopia, this study highlights the importance of more surveillance studies to increase our understanding of the dynamics of these genes.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12936-022-04306-3.

Acknowledgements
We would like to thank the patients in Amhara region for participating in this project. We would also like to thank all the professionals involved in this project.
Author contributions
IMF and PB conceived the original idea. MY, MA and AA coordinated and conducted the collection of samples. IMF, LG and VG conducted molecular analysis. IMF, JM and MM carried out data analysis. IMF, MW and PB drafted the manuscript. All authors contributed substantially to the manuscript review and approved its final version. PB, AB, WM, and JM are the guarantors. All authors read and approved the final manuscript.

Funding
This work was supported by the project TRPY 111/18 funded by the Institute of Health Carlos III, and IMF received a research fellowship from the University of Alcalá that enables her to develop this study.

Availability of data and materials
The datasets supporting the conclusions of this article are included within the article and its additional files, and the databases can be made available upon request to qualified researchers.

Declarations
Ethics approval and consent to participate
The Health Bureau of Amhara National Regional State Ethics Committee approved the collection of samples, their transportation, and their molecular and genetic analysis (No. Mo/go/tr/1/162/07). All participants signed an informed consent to participate in the study.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
1. Department of Biomedicine and Biotechnology, School of Pharmacy, University of Alcalá, Alcalá de Henares, Madrid, Spain. 2. Malaria and Neglected Diseases Laboratory, National Centre of Tropical Medicine, Institute of Health Carlos III, Madrid, Spain. 3. Public Health and Epidemiology Research Group, School of Medicine, University of Alcalá, Alcalá de Henares, Madrid, Spain. 4. College of Medicine and Health Sciences, Bahir Dar University, Bahir Dar, Ethiopia. 5. CIBERNET-CIBER Infectious Diseases (CSCI), Madrid, Spain. 6. Mundo Sano Foundations, Institute of Health Carlos III, Madrid, Spain. 7. Department of Infection Biology, London School of Hygiene and Tropical Medicine, London, UK.

Received: 28 December 2021 Accepted: 29 September 2022

Published online: 08 October 2022

References
1. WHO. World malaria report 2021. Geneva: World Health Organization; 2021. https://cdn.who.int/media/docs/default-source/malaria/world-malaria-reports/9789240040494-eng.pdf?sfvrsn=84fa6712_5&download=true.
2. Wang D, Chaki P, Macha Y, Gavana T, Michael MG, Khatibu R, et al. Application of community-based and integrated strategy to reduce malaria disease burden in southern Tanzania: the study protocol of China­UK­Tanzania pilot project on malaria control. Infect Dis Poverty. 2019;8:4.
3. Incardona S, Bell D, Campillo A, Cunningham J, Aney F, Fandeur T, et al. Keep the quality high: the benefits of lot testing for the quality control of malaria rapid diagnostic test. Malar J. 2020;19:247.
4. Kavuvaana MJ, Azzam SE, Rockabrand DM. Malaria rapid diagnostic tests: literary review and recommendation for a quality assurance, quality control algorithm. Diagnostics. 2021;11:768.
5. 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.
6. Maltha J, Gamboa D, Bendezu J, Sanchez L, Cronos L, Gilbert P, et al. Rapid diagnostic tests for malaria diagnosis in the Peruvian Amazon: impact of pfhrp2 gene deletions and cross-reactions. PloS ONE. 2012;7: e43094.
7. Beadle C, Long GW, Weiss WR, McElroy PD, Maret SM, Olozo AJ, et al. Diagnosis of malaria by detection of Plasmodium falciparum HRP-2 antigen with a rapid dipstick antigen-capture assay. Lancet. 1994;343:564–8.
8. Poti KE, Sullivan DJ, Dondorp AM, Woodrow CJ. HRP2: transforming malaria diagnosis, but with caveats. Trends Parasitol. 2020;36:112–26.
9. Kozycki CT, Ulusilma N, Rulisa SM, Mwikagara EJ, Musabiyima JP, Habmana JP, et al. False-negative malaria rapid diagnostic tests in Rwanda: impact of Plasmodium falciparum isolates lacking hrp2 and declining malaria transmission. Malar J. 2017;16:123.
10. Berhane A, Anderson K, Mihreteab S, Gheyi K, Rogier E, Mohamed S, et al. Major threat to malaria control programs by Plasmodium falciparum lacking histidine-rich protein 2. Eritrea Emerg Infect Dis. 2018;24:662–70.
11. Berhane A, Russom M, Bahta I, Hagos F, Ghirmay M, Uqubay S. Rapid diagnostic tests failing to detect Plasmodium falciparum infections in Eritrea: an investigation of reported false negative RDT results. Malar J. 2017;16:105.
12. Verma AK, Bharti PK, Das A. HRP2 deletion: a hole in the ship of malaria elimination. Lancet Infect Dis. 2018;18:826–7.
13. Baker J, Ho MF, 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.
14. Smith GP. Evolution of repeated DNA sequences by unequal crossover. Science. 1976;191:528–35.
15. Baker J, McCarty C, 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 PFHR2P-based rapid diagnostic tests. J Infect Dis. 2005;192:192.
16. Wilkie N, Zimmerman PA, Mehlotra RK. Plasmodium falciparum histidine‑rich protein 2 gene variation in a malaria‑endemic area of Papua New Guinea. Am J Trop Med Hyg. 2018;99:697–703.
17. Lee N, Baker J, Andrews KS, 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;44:2737–8.
18. Leow C, Jones M, Cheng Q, Mahler S, McCarthy J. Production and characterization of specific monoclonal antibodies binding the Plasmodium falciparum diagnostic biomarker, histidine-rich protein 2. Malar J. 2014;13:277.
19. Plucinski M, Aidoo M, Rogier E. Laboratory detection of malaria antigens: a strong tool for malaria research, diagnosis, and epidemiology. Clin Microbiol Rev. 2021;34: e0025020.
20. Gatton ML, Dunn J, Chaudhury A, Ciketic S, Cunningham J, Cheng Q. Implications of parasites lacking Plasmodium falciparum histidine-rich protein 2 on malaria morbidity and control when rapid diagnostic tests are used for diagnosis. J Infect Dis. 2017;215:1156–66.
21. Sepúlveda N, Phelan J, Diez-Benavente E, Campino S, Clark TG, Hopkins H, et al. Global analysis of Plasmodium falciparum histidine-rich protein-2 (pfhrp2) and pfhrp3 gene deletions using whole-genome sequencing data and meta-analysis. Infect Genet Evol. 2018;62:211–9.
22. Feleke SM, Reichert EN, Mohammed H, Bhrane 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.
23. Akinyi S, Hayden T, Gamboa D, Torres K, Bendezu J, Abdallah JF, et al. Multiple genetic origins of histidine-rich protein 2 gene deletion in Plasmodium falciparum parasites from Peru. Sci Rep. 2013;3:2797.
24. Fontecha G, Pinto A, Escobar D, Matamoros G, Ortiz B. Genetic variability of Plasmodium falciparum histidine-rich proteins 2 and 3 in Central America. Malar J. 2019;18:31.
25. WHO. World malaria report 2020. Geneva: World Health Organization; 2020. https://www.who.int/publications/i/item/9789240015791. Accessed 13 Dec 2020.
26. Ministry of Health, Ethiopia. Ethiopia malaria elimination strategic plan: 2021–2025. Addis Ababa, 2021.
27. Ministry of Health, Ethiopia. Ethiopia national malaria indicator survey 2015. Addis Ababa, 2015.
28. Taffeke HS, Hemming-Schoedler E, Koefpfl C, Tesfaye G, Lee M, Kazura J, et al. Malaria epidemiology and interventions in Ethiopia from 2001 to 2016. Infect Dis Poverty. 2018;7:103.
29. 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.

30. Golasia J, Messele A, Amambua-Ngwa A, Svedberg G. High prevalence and extended deletions in Plasmodium falciparum hrp2/3 genomic loci in Ethiopia. PLoS ONE. 2020;15:e241807.

31. Ndueri D, Kimani F, Thiongo 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.

32. Plowe CV, Djimde A, Bouare M, Doumbo O, Wellems TE. Pyrimethamine and proguanil resistance-conferring mutations in Plasmodium falciparum dihydrofolate reductase: polymerase chain reaction methods for surveillance in Africa. Am J Trop Med Hyg. 1995;52:565–8.

33. Berzosa P, Gonzalez-V, Taravillo L, Mayor A, Romay-Barja M, Garcia L, et al. First evidence of the deletion in the pfhrp2 and pfhrp3 genes in Plasmodium falciparum from Equatorial Guinea. Malar J. 2020;19:199.

34. Miguel-Otero M, Jirim A, Ta-Tang H, Lanza M, Hisam S, Rubio JM. Nested multiplex PCR for identification and detection of human Plasmodium species including Plasmodium knowlesi. Asian Pac J Trop Med. 2017;10:299–304.

35. Rubio JM, Post RJ, Van Leeuwen WM, Henry MC, Linderberg G, Hommel M. Alternative polymerase chain reaction method to identify Plasmodium species in human blood samples: the semi-nested multiplex malaria PCR (SnaM-PCR). Trang Trop Med Hyg. 2002;96(Suppl.1):S199.

36. Bharti PK, Chandel HS, Ahmad A, Krishna S, Udhayakumar V, Singh N. Prevalence of pfhrp2 and/or pfhrp3 gene deletion in Plasmodium falciparum population in eight highly endemic states in India. PLoS ONE. 2016;11:e0157949.

37. Kobayashi T, Sikalima J, Parr JB, Chaponda M, Stevenson JC, Thuma PE, et al. The search for Plasmodium falciparum histidine-rich protein 2/3 deletions in Zambia and implications for Plasmodium falciparum histidine-rich protein 2-based rapid diagnostic tests. Am J Trop Med Hyg. 2019;100:842–5.

38. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 1994;22:4673.

39. Weaver S, Shank SD, Spielman SJ, Li M, Muse SV, Kosakovsky Pond SL. Datamonkey 2.0: a modern web application for characterizing selective and other evolutionary processes. Mol Biol Evol. 2018;35:773–7.

40. Bharti PK, Chandel HS, Ahmad A, Krishna S, Udhayakumar V, Singh N. Prevalence of pfhrp2 and/or pfhrp3 gene deletion in Plasmodium falciparum population in eight highly endemic states in India. PLoS ONE. 2016;11:e0157949.

41. Scherf A, Mattei D. Cloning and characterization of chromosome break-point on the surveillance of pfhrp2 gene deletions. Elife. 2019;8:e40339.

42. Alemayehu GS, Messele A, Blackburn K, Lopez K, Lo E, Janies D, 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.

43. Mussa A, Talib M, Mohamed Z, Hajissa K. Genetic diversity of Plasmodium falciparum histidine-rich protein 2 and its effect on the performance of PfHRP2-based rapid diagnostic tests. BMC Res Notes. 2019;12:334.

44. Bakari C, Jones S, Subramaniam G, Mandara CI, Chiduo MG, Rumisha S, et al. Malaria infection in northern Ethiopia, 2015. Malar J. 2022;21:70.

45. Dejazmach Z, Alemayehu GS, Deme AB, Park DJ, Bei AK, Sarr O, Badiane AS, Gueye PEHO, et al. Analysis of PfHRP2/pLDH malaria RDT with microscopy and nested PCR methodology in Harari Region, Eastern Ethiopia. Infect Dis Poverty. 2020;9:160.

46. WHO. Surveillance template protocol for pfhrp2/pfhrp3 gene deletions. Geneva: World Health Organization; 2020.

47. Leonard CM, Assefa A, McCaffery JN, Herman C, Plucinski M, Sime H, et al. Investigation of Plasmodium falciparum pfhrp2 and pfhrp3 gene deletions and performance of a rapid diagnostic test for identifying asymptomatic malaria infection in northern Ethiopia, 2015. Malar J. 2021;21:70.

48. Molina-de la Fuente I, Herrador Z, Benito A, Berzosa P. Impact of Plasmodium falciparum pfhrp2 and pfhrp3 gene deletions on malaria control worldwide - a systematic review and meta-analysis. Malar J. 2021;20:276.

49. Sooloo SY, Alakara A, Namrermbe E, Kiggundu M, Nankabirwa JI, Ruhamy-ankaka E, et al. Determination of pfhrp2 and pfhrp3 genes were uncommon in rapid diagnostic test-negative Plasmodium falciparum isolates from Uganda. Malar J. 2021;20:4.

50. Menegon M, LEpiscopia M, Nurahmed AM, Talha AA, Nour BM, Severini C. Identification of Plasmodium falciparum isolates lacking histidine-rich protein 2 and 3 in Eritrea. Infect Genet Evol. 2017;55:131–4.

51. Scherf A, Wiette D. Cloning and characterization of chromosome break-points of Plasmodium falciparum: breakage and new telomere formation occurs frequently and subtelomically in genomic regions. Nucleic Acids Res. 1992;20:1491–6.

52. Oj OJ, Slater HC, Verity R, Parr JB, Mwangadarlina MK, Tshifu A, et al. Modelling the drives of the spread of Plasmodium falciparum hrp2 gene deletions in sub-Saharan Africa. Elife. 2017;6:e25008.

53. Zhou M, Wang C, Otto TD, Oberstaller J, Liao X, Adapa SR, et al. Uncovering the essential genes of the human malaria parasite Plasmodium falciparum by saturation mutagenesis. Science. 2018;360:aaa97847.

54. 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.

55. Nderu D, Kimani F, Karanja E, Meyer CG, et al. PfHRP2/PfHRP3 diversity and the implications for PfHRP2-based malaria rapid diagnostic tests in Ghana. Malar J. 2016;15:101.

56. Dereme AB, Park DJ, Ou S, Saides D, Rekese AS, Gueye PHO, et al. Analysis of pfhrp2 genetic diversity in Senegal and implications for use of rapid diagnostic tests. Malar J. 2019;18:925.

57. Gutierrez F, Wirth N, Belli K, Pascual A, Albisu M, Camara C, et al. Pfhrp2 and pfhrp3 polymorphisms in Plasmodium falciparum isolates from Dakar, Senegal: impact on rapid malaria diagnostic tests. Malar J. 2013;12:34.

58. Wellin N, Mehlrotz RK, Howes RE, Rakotomanga TA, Ramboarina S, Ratimbao SA, et al. Insights into the performance of PfHR2/PlDH malaria RDT with microscopy and nested PCR methodologies. Parasitol Int. 2018;67:793–9.

59. Adida-Mensah O, Dinka B, Naogbe AM, Arneke S, Annani-Akoller M, Owredu E, et al. Plasmodium falciparum histidine-rich protein 2 diversity in Ghana. Malar J. 2020;19:256.

60. Becquet R, Wirth N, Belli K, Pascual A, Albisu M, Camara C, et al. Pfhrp2 and pfhrp3 gene deletions in selected regions of mainland Tanzania. Malar J. 2020;19:391.
71. Hawkes M, Conroy A, Opoka R, Namasopo S, Liles W, John C, et al. Use of a three-band HRP2/pLDH combination rapid diagnostic test increases diagnostic specificity for falciparum malaria in Ugandan children. Malar J. 2014;13:43.

**Publisher’s Note**
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.