

Genetic Variations of *Plasmodium Falciparum* Histidine-rich Protein 2 and 3 in Assosa Zone, Ethiopia: Its Impact on the Performance of Malaria Rapid Diagnostic Tests

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Abstract

Background: Rapid diagnostic tests (RDT) are commonly used for the diagnosis of *Plasmodium falciparum* malaria. However, false negative results of RDT caused by genetic variations of *P. falciparum* histidine-rich protein 2 and 3 genes (*pfhrp2/3*) threaten existing malaria case management and control efforts. The main objective of this study was to investigate the genetic variations of the *pfhrp2/3* genes.

Methods: A cross-sectional study was conducted from malaria symptomatic individuals in 2018 in Assosa zone, Ethiopia. Finger prick samples were collected for RDT and microscopic examination of thick and thin blood films. Dried blood spots (DBS) were used for genomic parasite DNA extraction and molecular detection. Amplification of parasite DNA was made by quantitative PCR. DNA amplicons of *pfhrp2/3* were purified and sequenced.

Results: The PfHRP2 repeat type isolates were less conserved compared to the PfHRP3 repeat type. A total of eleven and eight different PfHRP2 and PfHRP3 amino acid repeat types were identified, respectively. Type 1, 4 and 7 repeats were shared by PfHRP2 and PfHRP3 isolates. Type 2 repeats were found only in PfHRP2, while types 16 and 17 were found only in PfHRP3 with a high frequency in all isolates. 18 novel repeat types were found in PfHRP2 and 13 novel repeat types were found in PfHRP3 in single or multiple copies per isolate. The positivity rate for PfHRP2 RDT was high, 82.9% in PfHRP2 and 84.3 % in PfHRP3 sequence isolate at parasitemia levels >250 parasites/ μ l. Using the Baker model, 100 % of the isolates in group A and 73.7% of the isolates in group B were predicted to be detected by PfHRP2 RDT at parasitemia level > 250 parasite/ μ l.

Conclusion: The findings of this study indicate the presence of different PfHRP2 and PfHRP3 amino acid repeat including novel repeats in *P. falciparum* from Ethiopia. These results indicate that there is a need to closely monitor the performance of PfHRP2 RDT associated with the genetic variation of the *pfhrp2* and *pfhrp3* gene in *P. falciparum* isolates at the country-wide level.

Background

Malaria caused by *Plasmodium falciparum* (*P. falciparum*) has remained a life-threatening infectious disease despite significant advances made towards malaria control and elimination in most malaria endemic countries in the world [1]. *P. falciparum* is the main cause of complicated malaria among human malaria parasites. The genetic variations in the genome of *P. falciparum* have the potential to make pathogenesis virulent and challenge the accuracy of diagnosis [2]. One of the strategic pillars to reduce the morbidity and mortality associated with falciparum malaria is the provision of rapid and accurate malaria diagnostic tools in malaria-endemic settings[3].

Rapid diagnostic tests (RDTs) are widely and routinely used in remote and resource limited settings. Currently available malaria RDTs utilize parasite-specific antigens produced by malaria parasites for diagnosis [4]. The most commonly used biomarkers in antigen-based malaria RDTs are lactate dehydrogenase (LDH), aldolase, and histidine-rich protein 2 (HRP2) [5]. Among these malaria RDT antigens, *P. falciparum* histidine-rich protein 2 (PfHRP2) is a specific biomarker used for diagnosis of *P. falciparum* infections [6]. The PfHRP3 antigen produced by *P. falciparum* has been shown to share high homology with PfHRP2 [7]. Therefore, the PfHRP3 antigen plays a substantial role in the detection of *P. falciparum* infections by PfHRP2-based RDTs [8].

The *pfhrp2* and *pfhrp3* genes are located in the subtelomeric region of chromosome 8 and 13, respectively. Both *pfhrp2* and *pfhrp3* genes consist of a single intron and two exons[7, 9]. Exon 2 in both *pfhrp2* and *pfhrp3* encodes amino acids which exhibit high levels of homology, suggesting that these genes were originated by duplication and divergence from a common ancestral sequence [7, 10]. The amino acid sequences of PfHRP2 and PfHRP3 contain several tandem copies of alanine and histidine-rich repeat. The PfHRP2/3 amino acid sequences with multiple repeat of AHH and AHHAAD contain approximately 35% histidine (H), 40% alanine (A), and 12% aspartate (D)[7]. Baker et al. showed several epitopes in PfHRP2 and PfHRP3 such as type 2 (AHHAHHAAD), type 4 (AHH), and type 7(AHH AAD) that are targeted by monoclonal antibodies against PfHRP2 and PfHRP3 antigens based on the PfHRP2 RDTs [11, 12].

However, frequent recombinations that occur at the subtelomeric regions contribute to vast genetic variation in the *Pfhrp2* and *pfhrp3* genes [13–15]. Recent studies from several African and South American countries showed that extensive variation in the amino acid sequences of PfHRP2 and PfHRP3 could influence the frequency of the respective epitopes recognized by monoclonal antibodies [12, 15, 16]. Furthermore, deletions of the *pfhrp2/3* gene cause a lack of target antigen in the diagnosis of *P. falciparum* malaria, as indicated in previous studies [17–19]. Genetic variation and deletion of PfHRP2 and PfHRP3 amino acid sequence could cause false negative testing using PfHRP2-based RDTs [20, 21]. Ultimately, inaccurate diagnosis could threaten ongoing efforts in malaria control and prevention [22].

While there is no data on the genetic variation of *pfhrp2/3* gene in Assosa zone, northwest Ethiopia, information on the abundance of PfHRP2 and PfHRP3 peptides repeats in *P. falciparum* isolates from geographically distinct regions is vital to monitor the diagnostic performance of PfHRP2 RDTs. Thus, the aim of this study was to determine the extent of genetic variation of *pfhrp2* and *pfhrp3* in clinical isolates, where *P. falciparum* is highly prevalent and PfHRP2 RDT is used for front-line diagnosis.

Methods

Study area and Period

This study was carried out during the low and high transmission seasons in four selected health facilities: Assosa, Bambasi, Kurmuk and Sherkole Health Centres in Assosa Zone, Benishangul-Gumuz regional state, northwest Ethiopia, April to December 2018. The study area has a high malaria transmission setting. The Assosa Zone is located on the border of Sudan, where transboundary transmission of malaria could occur. The map of the study area is indicated in additional file 1.

Study Design, Sample Size and Sampling Technique

A cross-sectional health facilities based study was conducted to assess genetic variation of *pfhrp2/3* in clinical isolates. The study populations were residents of the Assosa Zone and the source of the population was all patients with clinical suspicion of malaria aged ≥ 5 years in the selected health facilities during the study period.

The sample size was calculated based on the single population proportion formula $n = z^2p(1-p)/d^2$ [23]; Where, n = the sample size, $z = 1.96$ at 95% confidence interval (IC), d = margin of error at 5%, p (expected malaria prevalence rate) is 40% prevalence of symptomatic malaria in a hospital study of the region [24]. As a result, the sample size calculated with 10% non-response was 406 study participants. A total of 812 study participants were involved in this study, 406 study participants in low and 406 study participants in high transmission seasons.

The study area, Assosa Health Centre, Bambasi Health Centre, Kurmuk Health Centre and Sherkole Health Centre, was selected using a simple random sampling technique among eight districts in the Assosa zone. Then, allocation of the study participant to each selected health centre was performed based on proportion of confirmed malaria case in each selected district/woreda.

Sample Collection

In a single finger-prick, capillary blood samples were collected from malaria suspected individuals for microscopy, malaria RDTs as well as dried blood spots (DBSs) for molecular assay. Over all work flow of this study indicated in Fig. 1.

Microscopy and Malaria RDT

The CareStart™ malaria RDTs (Pf/PV HRP2/PLDH) were used following manufacturer's instructions. Thick and thin blood smears were stained with a 10% buffer-diluted Giemsa stain working solution for microscopic detection and the measurement of parasite density according to WHO recommendations [25].

Parasite DNA extraction and molecular analysis

Genomic DNA was extracted from DBSs using the chelex-saponin method as described previously [26]. *Plasmodium falciparum* identification was confirmed by SYBR Green quantitative PCR (qPCR) assay after amplification of 18S ribosomal RNA gene using species-specific primers [27]. After confirming *P. falciparum* positive samples, all PCR reaction of *pfhrp2* exon2 and *pfhrp3* exon3 were performed at 25ul total with 2x Promega Hot Start Master Mix (Promega Corporation, Madison, USA), 0.4ul each of forward and reverse primers, and 3ul of extracted template DNA using PCR conditions previously described [18, 22, 28]. PCR product of *pfhrp2* exon2 and *pfhrp3* exon2 were separated by electrophoresis on a 2% agarose gel and visualized in a UV transilluminator and size of the expected amplicons compared to 1kb DNA ladder. Primer sequences, PCR conditions, and expected amplicon size of *pfhrp2* exon2 and *pfhrp3* exon2 are provided in additional file 2.

Sequencing analyses

A total of 48 *pfhrp2* exon2 and 88 *pfhrp3* exon2 high quality sequence data were included for molecular analysis of *pfhrp2/3* genetic variation. *Pfhrp2/3* sequence of this study deposited in the NCBI Gen Bank database (with Accession number: MI050658-MI050792). All amplicons were cleaned using Exosap and sequenced using Sanger technology with ABI BigDye™ Terminator v 3.1 chemistry (ThermoFisher, Santa Clara, CA) and ran on a 3130 Genetic Analyzer (ThermoFisher, Santa Clara, CA). Sequences were then cleaned and analyzed using Codon Code Aligner Program V.6.0.2 (Codon Code Corporation, Centerville, MA). Nucleotide sequences were inputted into the Expasy Translate Tool (Swiss Institute of Bioinformatics Resource Portal) and translated into corresponding amino acids sequence using the correct open reading frame. The amino acid repeat sequences in *pfhrp2* and *pfhrp3* were given a numeric code system as describe previously [8, 11].

Data analysis

The proportions of each amino acid repeat of PfHRP2 and PfHRP3 in the *P. falciparum* isolates were analyzed using Statistical Package for Social Sciences (SPSS) version 20. The association between PfHRP2 RDT results (sequenced samples could be RDT positive or negative) and certain group of amino acid repeat was tested by Chi square and Fisher's exact test. P-values < 0.05 were interpreted as statistically significant.

Bakers' model used for the prediction of PfHRP2 RDT sensitivity, the sequences of PfHRP2 were classified into four groups based on combined length of types 2 × type 7 repeats. They include group A (≥ 100 , very sensitive), group B (50–99, sensitive), group C (< 43 , non-sensitive) and group I (44–49, borderline). The PfHRP2/3 amino acid sequences obtained from Ethiopia were compared with isolates from other countries based on the sequences deposited in the National Center for Biotechnology Information's (NCBI) GenBank using Basic Local Alignment Search Tool for Protein analysis (BLASTP).

Results

Genetic variation of PfHRP2 and PfHRP3 amino acids repeats

The length of *pfhrp2* exon2 sequences varied between 453 and 873 base pair (bp) ((ranged from 150 to 290 amino acids (aa)) among the 48 PfHRP2 sequenced samples. A total of 11 different PfHRP2 amino acid repeat types were identified (Table 1).

Table 1
Base pair length, amino acid length, number of known repeat type, and novel variants of PfHRP2 and PfHRP3 by study sites.

District	PfHRP2					PfHRP3				
	Total No	Mean			Total No	Mean				
Sequence analyzed	Known repeat type	Novel variants	Base pair length (range)	Amino acid length(range)	Sequence analyzed	Known repeat type	Novel variants	Base pair length (range)	Amino acid length(range)	
Sherkole	24	11	14	542(453–825)	239(150–283)	34	8	7	555(466–636)	182(154–211)
Bambasi	15	11	2	735(554–873)	244(184–289)	32	8	5	593(493–646)	196(164–215)
Kurmuk	7	10	2	733(638–872)	244(212–290)	20	8	7	573(461–654)	190(153–217)
Assosa	2	9	1	787(770–805)	230(212–247)	2	8	0	515(464–567)	171(154–188)

Nearly 65% (31/48) of the distinct PfHRP2 amino acid sequence occurred only once, while five PfHRP2 amino acid sequence patterns (I-V) were found in more than one isolate (Fig. 2). The structural organizations of the amino acid repeats were found at different positions of the PfHRP2 amino acid sequences. PfHRP2 amino acid sequences of all the isolates started with a type 1 repeat, 89.6% of the isolates ended with a type 10 repeat (occurred in five PfHRP2 patterns and distinct PfHRP2 sequence), and 10.4% of the isolates with a type 12 repeat (occurred only in four distinct PfHRP2 isolates). About 48% (23/48) of the isolates had a PfHRP2 repeat motif composed of types 2, 3, 5, 7, 8, 2, and 7, and this motif was absent in all PfHRP2 patterns. About 15% (7/48) of the isolates had a PfHRP2 repeat motif composed of types 7, 8, 2, and 7, and this motif was found in all PfHRP2 patterns except pattern III (Fig. 2). Types 2 and 7 were the most frequent repeats among isolates and were broadly distributed in PfHRP2 amino acid sequence.

The type and frequency of each PfHRP2 amino acid repeat varied among parasite isolates at study sites. Each PfHRP2 amino acid sequence contains 15–36 repeats. Repeat types 1, 2 and 7 were found in 100% of the isolates, repeat types 6 and 10 were found in 95.8% of the isolates, and type 5, type 3 and type 8 were found in 75-91.7% of the isolates being sequenced. Lower frequencies of PfHRP2 amino acid repeats were observed in type 4 (found in 20 isolates; 41.7%), as well as types 12 and 13 (found in 5 isolates; 10.4%). By contrast, types 9 and 11 were not identified in any PfHRP2 amino acid sequences in this study (Table 2).

Table 2
Distribution of PfHRP2 and PfHRP3 Amino acids repeat in *P. falciparum* isolates in Assosa zone, Northwest Ethiopia

Type of repeat	*Amino acid repeat sequence	PfHRP2 (n = 48)		PfHRP3 (n = 88)	
		No (%)	Min to Max number	No (%)	Min to Max number
1	AHHAHHVAD	48(100)	1–5	83(94.3)	0–3
2	AHHAHHAAD	48(100)	1–15	0(0.0)	0
3	AHHAHHAAY	42(87.5)	0–2	0(0.0)	0
4	AHH	20(41.7)	0–3	78(88.6)	0–1
5	AHHAHHASD	36(75.0)	0–1	0(0.0)	0
6	AHHATD	46(95.8)	0–6	0(0.0)	0
7	AHHAAD	48(100)	3–13	85(96.6)	0–2
8	AHHAAY	44(91.7)	0–2	0(0.0)	0
9	AAAY	0(0.0)	0–0	0(0.0)	0
10	AHHAAHHATD	46(95.8)	0–2	0(0.0)	0
11	AHN	0(0.0)	0–0	0(0.0)	0
12	AHHAAHHEAATH	5(10.4)	0–1	0(0.0)	0
13	AHHASD	5(10.4)	0–1	0(0.0)	0
14	AHHAHHATD	0(0.0)	0	0(0.0)	0
15	AHHAHHAAN	0(0.0)	0–0	84(95.5)	0–2
16	AHHAAN	0(0.0)	0–0	88(100)	7–16
17	AHHDG	0(0.0)	0–0	88(100)	1–8
18	AHHDD	0(0.0)	0–0	87(98.9)	0–4
19	AHHAA	0(0.0)	0–0	0(0.0)	0
20	SHHDD	0(0.0)	0–0	87(98.9)	0–2
21	AHHAHHATY	0(0.0)	0–0	0(0.0)	0
22	AHHAHHAGD	0(0.0)	0–0	0(0.0)	0
23	ARHAAD	0(0.0)	0–0	0(0.0)	0
24	AHHTHHAAD	0(0.0)	0–0	0(0.0)	0–0
*PfHRP2 and PfHRP3 amino acid repeat motif as describe by Baker et al [11]					
N = Total number of PfHRP2 and PfHRP3 amino acid sequence in <i>P. falciparum</i> isolates					
Min = Minimum number of amino acid repeat, Max = maximum number of amino acid repeat,					

Almost all PfHRP2 repeat types were found in all study sites with a slight difference in the frequency of repeat type within and between the study sites. Repeat types 1, 2 and 7 were found in all isolates of Sherkole, Bambasi, Kurmuk, and Assosa, while repeat types 3, 5, 6, 8 and 10 were found in 66–100% isolates among study sites. On the other hand, repeat types 4 and 12 were occurred in 6.7–62.5% of the isolates among study sites, while type 13 was found only in Sherkole (12.5%; 3/24) and Bambasi (13.3%; 2/15) (Additional File 3).

The length of the *pfhrp3 exon2* ranged from 461 to 654 bp (i.e., 153 to 217 aa) among the 88 sequenced samples. Of 88 PfHRP3 sequence isolates, eight different PfHRP2 amino acid repeat types were identified (Table 1), while eight distinct PfHRP3 patterns detected in more than one isolate (Fig. 3). The PfHRP3 sequence began with type 1 (present in PfHRP3 pattern I-V), type 16 (present in PfHRP3 pattern VII-VIII), and type 15 (present in PfHRP3 pattern VI) repeats in 93.2%, 4.6%, and 2.3% of the isolates, respectively; and ended with type 4 (present in PfHRP3 pattern I-IV and VII-VIII), type 17 (present in PfHRP3 pattern V), and type 18 (present in PfHRP3 pattern VI) repeats in 87.5%, 11.4%, and 1.1% of the isolates, respectively.

All isolates of the PfHRP3 amino acid sequence in this study contained a non-repeating sequence (ANHGFHFNLHDNNSHTLHHAKANACFDD) between types 7 and 20. Most of the isolates (96.6%; 85/88) had a similar unique PfHRP3 repeat motif with structural organization of repeat types 15, 16, 20, 17, and 18, and this motif was found in all PfHRP3 patterns except type VII (Fig. 3).

The total number of amino acid repeats and each repeat within the PfHRP3 sequence varied among the parasite isolates. Each PfHRP3 amino acid sequence consisted of 18–28 repeats. Repeats types 16 and 17 were found in 100% of the isolates, while types 1, 4, 7, 15, 18, and 20 were detected in more than 88% of the isolates. By contrast, types 2–3, 5–6, 8–9, 10–14, and 19 were not identified in the PfHRP3 amino acid sequences in this study. All PfHRP3 repeat types were found in all study sites with some difference in the frequency of the repeat type within and between study sites. Repeat types 16, 17, 18, and 20 were found in 100% of the isolates in Sherkole, Bambasi, Kurmuk and Assosa, while types 18 and 20 in 95% of the isolates in Kurmuk. On the other hand, repeat types 1, 4, 7, and 15 were found in 84.4–100% of the isolates among study sites (Additional File 3).

Analysis of Ethiopian PfHRP2 /3 amino acid sequences against the global isolates

BLAST analysis of PfHRP2/3 amino acid sequences were performed for each of the sequences obtained from Ethiopia to look for sequence similarities with other countries available from NCBI. PfHRP2 protein BLAST (BLASTP) analysis reveals a range of 88.50-98.82% similarity with *P. falciparum* isolates from Africa, India, and Myanmar, accession numbers from similar sequences are indicated in Additional file 4. Likewise, the BLASTP analysis of PfHRP3 reveals a range of 93.55% -100% similarity with *P. falciparum* isolates from Kenya and India, accession numbers from similar sequences are indicated in Additional file 5.

Comparison of Ethiopian Novel variants of PfHRP2/3 repeat types with global.

A total of 18 novel variants of PfHRP2 repeat types were found in this study (Table 3). Of these novel PfHRP2 amino acid repeat sequences, 15 were not reported previously. Though type 7 (AHHDD) and type 10 (AHHAATHHATD, AHHA AAHHAND) repeats were reported previously (Additional file 6), they occurred at a very low frequency. Similarly, 13 new PfHRP3 repeat types were identified among the Ethiopian isolates. Among them, 12 were not previously reported. Only type 20 (SHHDG) was previously reported (Additional file 7) and it occurred at high frequency among the Ethiopian isolates.

Table 3
List of Novel repeat /variants of PfHRP2 and PfHRP3 amino acid repeat types.

Type of repeat	Known Amino acid repeat	PfHRP2		PfHRP3	
		Novel repeat	Repeat frequency	Novel repeat	Repeat frequency
1	AHHAHHVAD	-	-	DHHAHHVAD	1
				PHHAHHVAD	4
				SPHAHHVAD	1
2	AHHAHHAAD	AHHAHHEAD	1		
		AHHAQHAAD	1		
		AHHAHHAHH	1		
		APDHHAHH	1		
		AHHAHVAAD	1		
		AHHAHHAAY	1		
3	AHHAHHAAY	AHHAHYAAY	1		
4	AHH			VHH	1
7	AHHAAD	AHHAVH	1		
		AHHADD	2		
		AHHAVD	1		
10	AHHAAAHHATD	AHAAAHHAT*	1		
		AHAAAADHHATD	1		
		AHAAAHQATD	1		
		AHAAAHHAND	1		
		AHAAAHHARD	1		
		AHHAADAHHATD	1		
		AHHAATHHATD	1		
12	AHAAAHHHEAATH	VHAAAHHHEAPLC	1		
15	AHHAHHAAN	-		AHHAHHADN	3
		-		AHHAHHVAN	1
16	AHHAAN	-		AHHAGD	4
		-		ALLAAN	1
		-		SHHAAN	1
		-			
18	AHHDD			EHHDE	1
				AHHDE	3
				ARHDD	1
20	SHHDD			SHHDG	7

The asterisks (†) show deletion position of amino acid code (double letter), **Note:** bold underline letter with yellow color indicate position of novel repeat with replacement of one or more amino acid compared to known repeat.

Effect of genetic variation of pfhrp2/3 on performance of RDTs

Of the 48 *P. falciparum* samples included in *Pfhrp2* exon2 sequence analysis, 72.9% (35/48) were detected as positive by PfHRP2 RDT. Among these PfHRP2 RDT positive samples, 82.9% (29/35) was found with a parasitemia level > 250 parasite / μ L. On the other hand, all PfHRP2 RDT negative samples (13/48) were submicroscopic (Additional file 8). Similarly, among 88 *P. falciparum* samples included in *Pfhrp3* exon2 sequence analysis, 79.5% (70/88) was detected by PfHRP2 RDT and 84.3% (59/70) of these positive samples had parasitemia levels > 250 parasites/ μ L (Additional file 9).

The performance of PfHRP2 RDT and the number of PfHRP2 amino acid repeat 1 (P = 0.944), repeat 2 (P = 0.456), repeat 6 (P = 0.95), repeat 7 (P = 0.486), and repeat 10 (P = 0.273) observed in the samples were not statistically associated with one another. Likewise, the performance of PfHRP2 RDT and variation of PfHRP3 amino acid repeat 1 (P = 0.928), repeat 4 (P > 0.05), repeat 7 (P > 0.05), repeat 15 (P > 0.05), repeat 18 (P = 0.436), and repeat 20 (P > 0.05) were not statistically associated. Interestingly, significant association was detected between performance of PfHRP2 and the number of PfHRP3 repeat 16 (P = 0.031) and repeat 17 (P < 0.001) (Additional file 10).

Amino acid change in PfHRP2 and PfHRP3 repeat types observed in this study might affect the performance of PfHRP2 RDT. Eighteen novel PfHRP2 and 13 novel PfHRP3 repeat types were identified within 13 PfHRP2 and 24 PfHRP3 sequence in the *P. falciparum* population, respectively. Of 13 PfHRP2 sequences, 38.5 % (5/13) that had the novel variants were detected negative by PfHRP2 RDT (Additional file 8). Of the 24 PfHRP3 sequences, 37.5 % (9/24) that had the novel variants were negative by PfHRP2 RDT (Additional file 9).

Prediction of PfHRP2 RDT sensitivity using Bakers' model.

According to the Baker's model, most of the isolates in this study was from group B (54.2%, 26/48) followed by group A (22.9%, 11/48). Among these isolates, 100 % (8/8) in group A and 73.7% (14/19) in group B were predicted to be detected by PfHRP2 RDT at parasitemia level > 250 parasite/ μ L. About 86% (6/7) of *P. falciparum* isolates in group C were sensitive to PfHRP2 RDT at parasitemia level > 250 parasites / μ L in this study (Table 4).

Table 4

Frequency of PfHRP2 sequence groups based on the Baker's model and PfHRP2 RDT results. (n= 48)

Frequency(N=48)		Prediction of PfHRP2 RDT Positivity	Microscopy >250 parasite/ μ L
Group	No (%)	No (%)	No (%)
A	11(22.9)	8(72.7)	8(100)
B	26(54.2)	19(73.1)	14(73.7)
C	10(20.8)	7(70.0)	6(85.7)
I	1(2.1)	1(100)	1(100)

PfHRP2 sequence isolates were classified into four groups according to the frequency number of types 2 \times type 7 repeats: group A (\geq 100, very sensitive), group B (50-99, sensitive), group C (<43, non-sensitive) and group I (44-49, borderline) as describe previously (Baker et al, 2010)

Discussion

The present study provide valuable information on genetic variations in the PfHRP2 and PfHRP3 amino acid repeat types, which could affect the performance of PfHRP2 RDT for *P. falciparum* diagnosis. The structural organization of PfHRP2 amino acid sequences was less conserved than PfHRP3 among the Ethiopia isolates. The sequence length and the number of amino acid repeat types of PfHRP2 and PfHRP3 in this study were comparable to those previously reported in Yemen [29] and Madagascar [16], but lower than those in Kenya [30] and Ghana [31] and higher than in India [32].

All PfHRP2 repeat types started with repeat type 1 in the Ethiopian *P. falciparum* isolates, similar to previous reports in Kenya [30], Yemen [29], Senegal [33], and outside Africa [8]. The majorities of the PfHRP2 repeat ended with type 10 repeat which showed discordance with the previous reports from Africa [29, 30, 33] and Asia [32]. On the other hand, a small proportion of the isolates (10.4%) ended with a type 12 PfHRP2 repeat coincide with reports from Senegal [34]. Two types of PfHRP2 repeat motifs structural organization repeat motifs (2, 3, 5, 7, 8, 2, and 7) and (7, 8, 2, 7), were identified [8, 30].

Eleven different PfHRP2 repeats and eight different PfHRP3 repeats were found at Sherkole, Bambasi, Kurmuk and Assosa with slight difference in the frequency of the repeat type within and between *P. falciparum* isolates in Ethiopia, similar to those reported in Africa [30, 31]

and worldwide [8]. The PfHRP2 repeat types observed in the present study were previously reported in Africa [29–31], Asia [8, 32], and America [35, 36]. BLAST analysis of the Ethiopia PfHRP2 and PfHRP3 amino acid sequences revealed the presence of similarities and shared identity with isolates from Kenya. Interestingly, the amino acid sequences similarities of PfHRP2 and PfHRP3 also extended beyond the border of Ethiopia, India, and Myanmar, which is also in agreement with a recent study from Ghana [31].

On the contrary, PfHRP2 repeat types obtained within this study showed certain difference from African and global reports in a number of ways. First, PfHRP2 type 12 repeat was found in a few isolates (10.5%) this did not align with previous studies from Kenya [30], Ghana [31] and central America [36]. However, studies from Senegal [34] showed the presence of type 12 repeats in a few isolates similar to the present study. Second, we did not find type 9 and 11 repeats isolates within our study which is consistent with most studies from African countries [29–31], but type 11 repeat was reported in a few isolates from Madagascar [16], while type 9 was reported from Senegalese isolates [34]. Third, types 14–27 were completely absent in all isolates in this study. These results disagree with other studies which reported the rare occurrence of type 14 from Nigeria [8] and Madagascar [16], and type 19 from Kenya [30]. The possible explanation for such varied distribution repeat types may be due to random mutation and local selection or directional spread of deleted strains of *pfhrp2/3* throughout the world [37].

Concerning PfHRP3, the majorities of sequences started with type 1 and ended with a type 4 repeats in the Ethiopia isolates, which agrees with previous studies [30, 32]. Moreover, all isolates of PfHRP3 amino acid sequence contained a singleton non-repeating sequence [11]. The findings of PfHRP3 repeat types in the present study are consistent with previous reports from Kenya [30], Yemen [29], Ghana [31], and globally [8]. On the other hand, type 2 repeat was absent in all isolates of PfHRP3 sequence in Ethiopia as well as other parts of Africa [29–31], but type 2 repeat has been reported in PfHRP3 in few isolates from Kenya [30] and India [32]. This variation observed in PfHRP2 and PfHRP3 repeat types might be due to differences in geographical and transmission settings [8, 38, 39], frequency of exposure of drug and level of immunity of study participants [40, 41], clinical versus asymptomatic study participants, and methods used for molecular analysis.

Interestingly, of all the novel repeat types identified in this study, 15 in PfHRP2 and 12 in PfHRP3 had not been reported elsewhere. Among all novel PfHRP2 repeat types, two unique repeat types (type 7-AHHDD, type 10-AHHAATHHATD) and one unique repeat type (type-AHHAAHHAND) were found with low frequency in Ethiopia and also in a previous study from Ghana [31] and Kenya [30]. Among all novel PfHRP3 repeat types, a unique repeat type (type 20-SHHDG) was detected with high frequency in Ethiopia as well as in Kenya [30], China-Myanmar border [42], and India [32]. The emergence of novel PfHRP2 and PfHRP3 repeat types at low frequency could be initiated by replacement or deletion of one or more amino acid repeat type [43–45].

Density of parasites and genetic variation *pfhrp2/3* gene are two most important factors that affect the performance of PfHRP2 RDT [8, 17, 22]. In this study, a high proportion of PfHRP2 RDT positive samples was observed with parasitemia > 250 parasites/ μ l, but substantial numbers of PfHRP2 RDT false negative samples were detected in submicroscopic infections similar to previous reports [8, 46].

Baker's model indicated that the Ethiopian isolates mostly belong to groups A and B were predicated to be detected by PfHRP2 RDT at parasitemia level > 250 parasite/ μ l and thus aligns with previous studies found in Senegal [34], Madagascar [16], and India [47]. Interestingly, most of *P. falciparum* isolates in group C were detectable by PfHRP2 RDT at parasitemia level \geq 200 parasite/ μ l. This finding partially disagrees with the Baker' model [11] that predicts if the length of repeat types 2 and 7 is below 43 (as in group C), it will alter detection sensitivity of PfHRP2 RDT and lead to false negative results [34].

In this study, the length of amino acid repeat in PfHRP2 (type 1, 2, 7) and shared repeats in PfHRP2 and PfHRP3 (type 1, 4, 7) were not statistically associated with the performance of PfHRP2 RDT, consistent with previous study [30]. Instead, PfHRP2 RDT positivity was significantly improved as the length of PfHRP3 repeat type 16 and 17 increased. Types 1, 4 and 7 were common repeat types in both PfHRP2 and PfHRP3 amino acid sequence, whereas types 16 and 17 were identified only in PfHRP3 in high frequency among all isolates. In line with this, PfHRP2 and PfHRP3 exhibit structural homology as exon 2 in both *pfhrp2* and *pfhrp3* encodes similar amino acids and cross reaction may play a role in the diagnosis of falciparum malaria [7, 12]. Moreover, novel PfHRP2 and PfHRP3 repeat variants detected in this study might influence the binding affinity to monoclonal antibody and affect the sensitivity of PfHRP2 RDTs [12]. As results, considerable proportions of false negative results were found in this study by PfHRP2 RDT, 38.5 % of PfHRP2 and 37.5 % of PfHRP3 sequence with novel variants.

Limitations

This study considered two limitations. First, samples represented a limited geographical area from Assoa, Ethiopia. Second, this study did not assess cross reactivity of the possible epitopes based on the amino acid repeat sequence of PfHRP2 and PfHRP2 using specific monoclonal antibodies.

Conclusion

The present study indicated, for the first time, the existence of genetic variability of PfHRP2 and PfHRP3 amino acid repeats including novel repeats in *P. falciparum* isolates within and between study sites in Ethiopia. There is a need to closely monitor the performance of PfHRP2 RDT and examine the distribution of novel repeat type and shared repeat in PfHRP2/3, and unique repeat found in PfHRP3 broadly in Ethiopia.

Abbreviations

RDT: Rapid diagnostic tests RDT; HRP2: Histidine-Rich Protein 2/3, *pfhrp2/3* gene: *P. falciparum* histidine-rich protein 2 and 3 genes ; DBS: Dried blood spots; PCR: polymerase chain reaction; qPCR: quantitative polymerase chain reaction, SPSS: Statistical Package for Social Sciences; WHO: World Health Organization; NCBI: National Center for Biotechnology Information's; BLASTP: Basic Local Alignment Search Tool for Protein analysis .

Declarations

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Authors' contributions

GA, AL, EL, DJ and LG conceived and designed the study. GA collected the sample. GA and KL performed laboratory analysis under the direction of LG, DJ and EL. All authors reviewed and approved this manuscript.

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Availability of data and materials

The datasets used and/or analyzed during in this study are included in this article and available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Ethical clearance was obtained from the institutional review board of the Aklilu Lemma Institute of Pathobiology, Addis Ababa University (Ref No ALIPB / IRB006 / 2017/18) and also from the Institutional Review Board of the University of North Carolina at Charlotte (IRB number 18-0451). Material transfer agreements were made between Addis Ababa University and the University of North Carolina at Charlotte. Blood samples were taken from finger prick after obtaining written informed consent and/or assent from parents or guardian for children. Health workers treated positive malaria cases according to national treatment guidelines.

Consent for publication

This study reports no individual person's data. There is no opposition to its presentation and/or publication. All authors have read and approved the manuscript for publication.

Competing interests

The authors declare that they have no conflict of interests.

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Figures

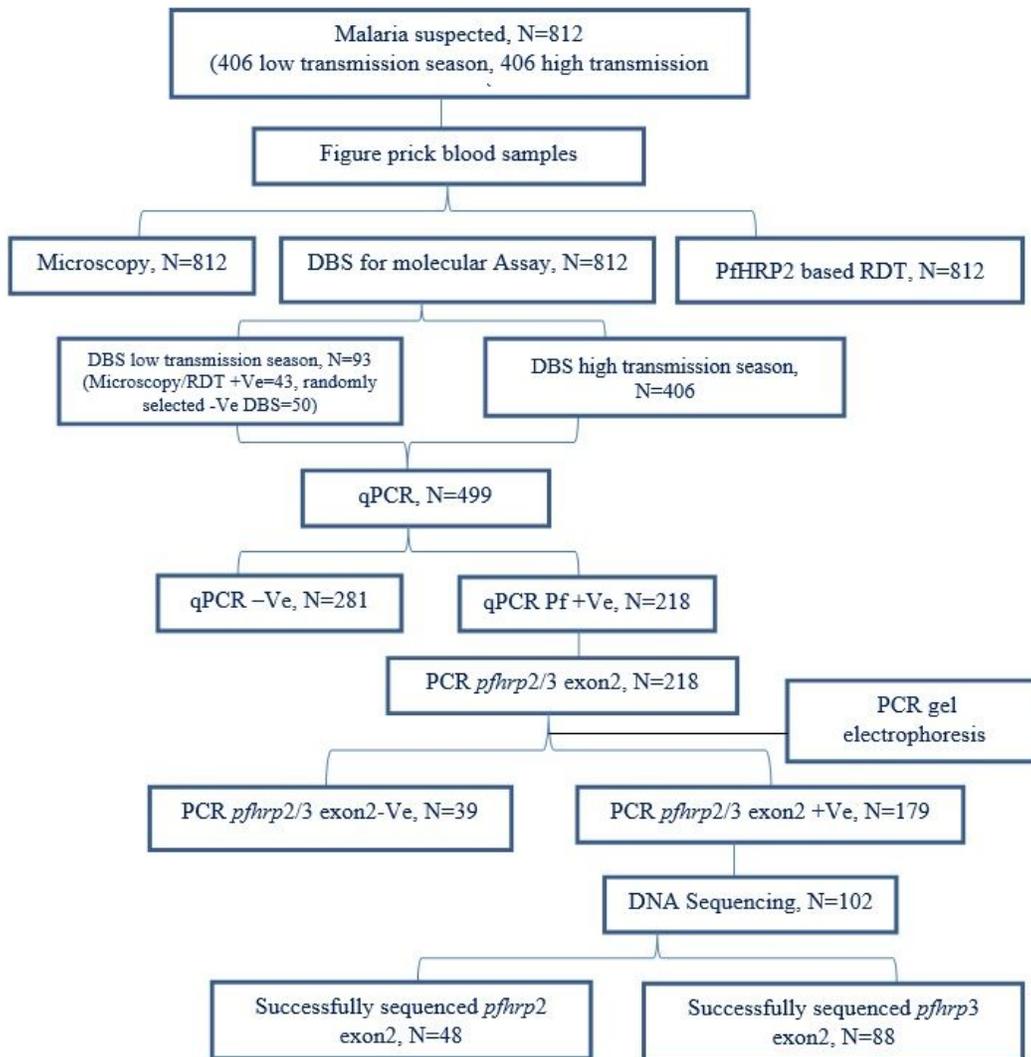


Figure 1

Study flow chart for molecular analysis of *pfhrp2/3* genetic variation. Note: DBS=Dried blood Spot, qPCR=quantitative PCR, Pf=*P.falciparum*, -Ve = negative, +Ve=Positive, N=number of samples,

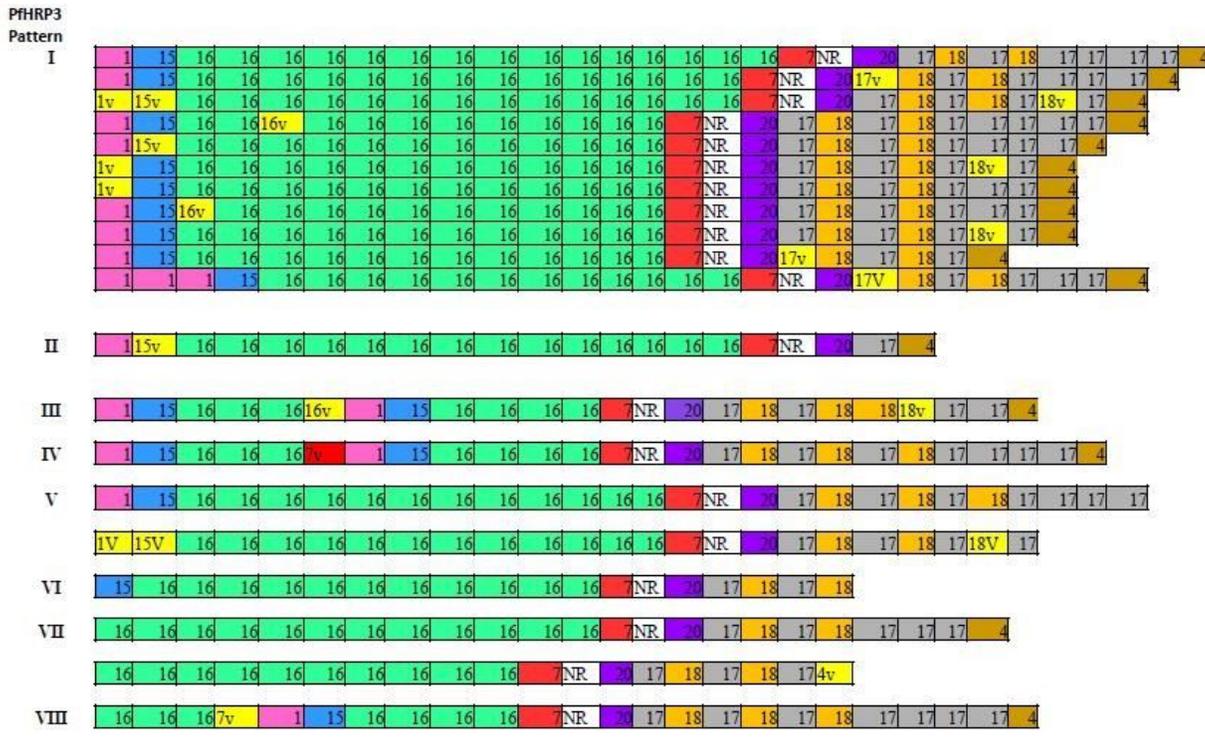
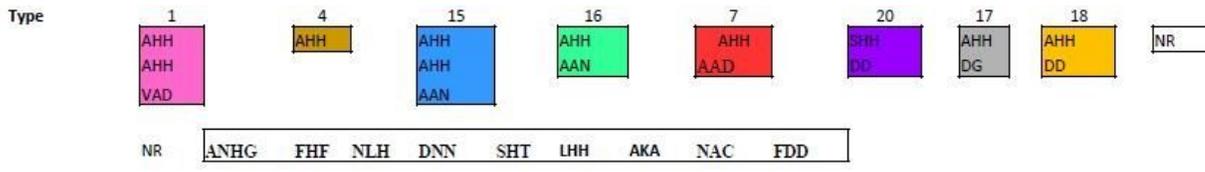


Figure 3
 PfHRP3 structural organization in Assosa isolates. Eight PfHRP3 patterns identified in more than one isolates. A total of eight PfHRP3 amino acid repeat type is differentiated by color block with their respective repeat type number. Unique variant repeat (V) display by yellow color. NR=Non-repetitive region

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