Extensive Deletion and Sequence Variation of Plasmodium Falciparum Histidine Rich Protein 2/3 (pfhrp2/3) Genes in Ethiopia: Implication for RDT-based Malaria Diagnosis and Control

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Research

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Abstract

Background: Despite remarkable malaria reduction in recent years, malaria remains a public health problem in Ethiopia. With the introduction of rapid diagnostic tests (RDTs), malaria diagnosis has been transformed. However, the Plasmodium falciparum histidine rich protein 2 (hrp 2) that is targeted by the most widely used RDTs is prone to genetic mutations and gene deletions as observed in recent years. Patients infected with P. falciparum malaria parasites with a deletion in hrp 2/3 gene locus would remain undetected and results in 'false-negatives', which are not treated. Undoubtedly, these untreated infected patients are at risk of developing complicated disease and may further fuel parasite transmission. Hence, molecular targeting of the region across exons and flanking genes has been used to provide greater confirmatory evidence of gene deletions. This study was initiated to determine pfhrp2 /3 gene deletions including the flanking regions.

Methods: A cross-sectional study was conducted to determine the prevalence of hrp 2/3 genes deletion.Finger-prick blood samples were collected from a total of 64 febrile patients attending Adama Malaria Diagnostic Centre in 2015. Thick and thin blood smears were prepared for microscopic slide readings, and parasitaemias were determined. Blood samples were spotted onto filter for parasite DNA extraction.

Results: From a total of 64 microscopically and PCR confirmed P. falciparum infections, 50 were successfully analyzed for deletion of pfhrp2 , pfhrp3 and flanking regions. Extensive deletions were observed in the pfhrp2 gene with all 50(100%) isolates presenting a deletion. This deletion extended downstream towards the Pf3D7 0831900 (MAL7PI.230) gene in 11/50 (22%) cases. In contrast, only 2/50 (4%) of samples had deletions for the upstream gene Pf3D7 0831700 (MALPI.228). Similarly, all isolates had deleted the pfhrp3 gene (100%) and this in 40% of isolates extended to the downstream flanking region Pf3D7 13272400 (MAL13PI.485) where 40% samples showed absence of this region. However, the deletion also extended toward the upstream region Pf3D7 081372100 (MAL13PI.475). The deleted pfhrp 3 genomic region also extended upstream to the region Pf3D7 081372100 (MAL13PI.475) with 49/50 (95%) of the isolates exhibiting absence of the locus.

Conclusion: As patient recruitment was not done on pfhrp 2/3-based RDTs, it is impossible to know if the isolates would test negative or positive in the absence of hrp 2/3 genes. Indeed, the sequence variation and high frequencies of deletion in pfhrp2 and pfhrp3 genes in Ethiopian isolates most likely will have a negative influence on the performance of currently used pfhrp2 RDTs. This study confirms the presence of that P. falciparum parasite population with extensive deletions of the pfhrp 2 and pfhrp3 genes in Ethiopia and this calls for a countrywide surveillance to determine the extent of these deletions and its effect on routine malaria diagnosis.

Keywords: Plasmodium falciparum , Histidine rich protein 2/3, Deletion, RDTs, Microscopy, Ethiopia

Introduction

In Ethiopia, *Plasmodium falciparum* and *Plasmodium vivax* co-exist with relative proportions of 0.60 and 0.40 of malaria cases, respectively [1, 2]. Nearly 68% of the landmass, where 60% of the population lives, is favourable for malaria transmission [1, 3]. Depending on the local malaria transmission intensity or endemicity class, the epidemiology of malaria varies geographically [4]. Like in many most of Africa, the incidence of malaria has been substantially reduced in Ethiopia which also reported a substantial 40% reduction in incidence by the year 2020 [5]. Ethiopia is on track to achieve the 2020 milestone by reducing the incidence of malaria by 40% and also aligns with the World Health Organization (WHO) Global Technical Strategy (GTS) by intensifying existing malaria control activities to eliminate malaria by 2030 [5]. Much of this can be accredited to the national malaria control and prevention strategies, which have introduced insecticide treated nets, insect residual spray and a change in drug policy. Essentially, rapid diagnostic tests (RDTs) have been introduced to improve early diagnosis of malaria in remote areas where microscopic examination of blood smears seems impractical.

RDTs are immune-chromatographic tests that are designed to detect malaria specific antigens such as lactate dehydrogenase or aldolase for pan-malaria diagnosis and *pfhrp2* for *P. falciparum* specific diagnosis [6]. Since the first report detailing the deletion of *pfhrp2* in Peru and its impending consequences was published in 2010, several studies have shown the global spread of parasites lacking *pfhrp2* gene and its flanking regions [7]. In Eritrea, *P. falciparum* lacking histidine-rich protein has become a major threat to malaria control programs [8, 9]. As they are not detected by *pfhrp*2/3-based RDTs and remain untreated, parasites carrying the *pfhrp*2/3 gene deletions have a fitness advantage posing a challenge to progress made in malaria control and elimination.

Particularly in South America, Peru has shown a high prevalence of these parasites with 41% deletion in *pfhrp2* and 70% deletion in *pfhrp3* gene, this was also confirmed by another study which showed an increase in prevalence from 20.7 to 40.6% within two years, in another region of the country [10]. Similarly, a study on *P. falciparum* outbreak on the northern Pacific coast of Peru (2010–2012) using 54 samples showed all 100% samples lacked the *pfhrp2* gene [11]. Following this, several countries have reported occurrence of *pfhrp2* and *pfhrp3* gene deletions [9, 12–21]. For highly malaria endemic Africa, the prevalence of the deletion from recent report have generally been low or absent. However, very high prevalence has been reported for Eritrea, which shares a border with Ethiopia. So far, no studies have been conducted in Ethiopia to survey or detect the status of *pfhrp2* deletions in the country. Undoubtedly, parasites with deleted *hrp*2/3 genes will be a significant setback for malaria elimination in this region since RDT-based diagnostics are a key pillar in effective malaria management and control. Following the WHO recommendation for *pfhrp2*/3 surveys and surveillance activities to target neighbouring countries where deletions have been reported, this study investigates the extent of *pfhrp2*/3 deleted *P. falciparum* parasites in Ethiopia. Molecular targeting the region across exons and flanking genes would provide greater confirmatory evidence of gene deletions.

Methods

Sample collection and diagnosis of malaria

Finger-prick blood samples were collected from a total of 64 febrile patients attending Adama Malaria Diagnostic Centre in 2015. Thick and thin blood smears were prepared for microscopic slide readings, and parasitaemias were determined. Blood samples were spotted onto filter for parasite DNA extraction.

Parasite Dna Extraction, Pcr Analysis And Dna Sequencing

Parasite DNA was extracted from blood spots on filter paper using chelex extraction methods as described elsewhere [22].

Detection and amplification of hrp2/3 genes

Successful amplification of *pfhrp2* and *pfhrp3* was made for a total of 50 samples confirmed both by microscope. Exon 2 of both genes was amplified to demonstrate the presence or absence of the *pfhrp2* and *pfhrp3* genes and their flanking regions using published protocols and primers (Additional file 3). Briefly, *pfhrp3* gene (475 to 485) was amplified using the following PCR conditions: denaturing at 95^0C for 3 minutes followed by 94^0C for 15 seconds (36 cycles), annealing at 55^0C for 15 seconds (35 cycles), elongation 60^0C for 1:3o minutes (36 cycles) and cooling at 16^0C. Amplification of the *pfhrp2* genes (475 to 485) was made as shown in Table 1.

Table 1
Outer and nested PCR conditions used to amplify *pfhrp2* gene.

***Hrp2* -Outer**	**Temp**	**Time**	**Process**
Step 1	95	3 min	Denaturation
Step 2	94	15 sec	
Step 3	55	15 sec	Annealing
Step 4	60	1:30 min	Elongation
Step 5	72	5 min	
Step 6	16	∞	Cooling
Hrp2**-Nested**	**Temp**	**Time**	**Process**
Step 1	95	3 min	Denaturation
Step 2	94	15 sec	
Step 3	55	15 sec	Annealing
Step 4	60	1 min	Elongation
Step 5	72	5 min	
Step 6	16	∞	Cooling

For amplification of *pfhrp2*, two different primer sets were used. The first one targets the repeat region which harbours most of the variable part of the gene (Additional file 3). The second primer set targets an intron region and for this primer set 5 samples gave adequate PCR products and their sequences were determined. Successful PCR products were purified by the GeneJet PCR Cleanup Kit from Thermo Fisher Scientific and sent for sequence determination at Eurofins genomics, Germany. Sequences were analysed by the 4peaks program (A. Griekspoor and Tom Groothuis, nucleobytes.com).

Ethical Issues

The study was approved by Aklilu Lemma Institute of Pathobiolohy, Addis Ababa University, Institutional review Board. Written consent and/or assent were obtained from each study participants.

Results

PCR confirmation of P. falciparum infections

Febrile patients travelled from various areas to Adama Malaria Diagnostic Centre for malaria diagnosis. The male: female ratio was 3.1:1. Participant's mean age was 25.2 years (range 11–48). A total of 50 specimens met the inclusion criteria for this study by yielding positive results for amplification for both 18S rRNA gene. Using 18S rDNA PCR, all 50 specimens were positive for *P. falciparum*. Additionally, all samples were amplified for the *pfmdr1* gene. The minimum parasite density reported by microscopy was 400 parasites/μL, which would indicate that this is the threshold for detection by the field microscopists.

Genetic deletion of pfhrp2 and pfhrp3 and their flanking genes

From the 64 microscopically and PCR confirmed *P. falciparum* infections, 50 were successfully analysed for *pfhrp2*, *pfhrp3* and flanking regions described above (Table 2). Extensive deletion was observed in the *pfhrp2* gene with all 50 (100%) isolates presenting a deletion. This deletion extended downstream towards the Pf3D7 0831900 (MAL7PI.230) gene in 11/50 (22%) of cases. In contrast only 2/50 (4%) of samples had deletions for the upstream gene Pf3D7 0831700 (MALPI.228).

The samples contained different AT repeat sequences (one sample with 10 repeats, and four samples with 17 repeats) (data not shown) suggesting that the deletion in this region did not involve the entire region. Indeed, the positive PCR products were from a small part covering an intron region where these repeats are found (Additional file 3).

The results for *pfhrp3* and flanking regions were comparable. Here, only the repeat region was analysed and all isolates had deletions in the *pfhrp3* gene (100%) and like *pfhrp2* this deletion extended towards the downstream flanking region Pf3D7 13272400 (MAL13PI.485) where 40% samples shown absence of this region. However, the extension of the deletion was more prevalent towards the upstream region Pf3D7 081372100 (MAL13PI.475), where 49/50 (95%) of the isolates exhibited absence of the loci. The extension of deletions in *pfhrp*2 and *pfhrp3* genes can be viewed from the Additional files 1 and 2.

By targeting six regions in the *hrp2/3* and flanking regions, different deletion patterns were observed in Ethiopian *P. falciparum* clinical samples (Table 2). A greater proportion of parasite isolates had deleted the gene located 3' of *pfhrp2*, PF3D7_0831900, compared to the flanking gene 5', PF3D7_0831700. In sharp contrast, the 5' flanking gene PF3D7_1372100 (upstream of *pfhrp3*) showed more deletions than the 3' flanking region PF3D7_1372400 region (downstream of *pfhrp3*). As such the most common pattern exhibited in the isolates was the presence of the the two flanking regions for *pfhrp*2 in combination with the downstream flanking region for *hrp3*. This was followed by isolates that had deleted the downstream flanking region for *hrp3* but retained the two flanking for *hrp*2. Notably, only one isolate showed the presence for all four flanking regions.

Table 2
Results of PCR amplification of *pfhrp2*, *pfhrp3* and their respective flanking genes in *P. falciparum* samples collected in Ethiopia.

No.	PF3D7_0831700	HRP2	PF3D7_0831900	PF3D7_1372100	HRP3	PF3D7_1372400
20	+	-	+	-	-	+
16	+	-	+	-	-	-
8	+	-	-	-	-	+
3	+	-	-	-	-	-
2	-	-	+	-	-	-
1	+	-	+	+	-	+
50						
The association between parasite density and *hrp*2 deletion was not evaluated due to the absence of *hrp2/hrp3* positive samples.						

Discussion

RDTs have become extremely essential for implementing early diagnosis and prompt effective treatment to substantially reduce the incidence rate of malaria in Africa. Nowadays, RDTs are the most widely used malaria diagnostics especially in areas where microscopic diagnosis is impractical for several reasons. However, variation in the performance of RDTs variation has been chronicled, probably driven by known deletion polymorphism targeting *pfhr2/hrp3* loci. However, the prevalence and dynamics of *pfhrp2* deleted strains has not been extensively investigated, especially in Africa. This study is one of the first reports showing an extensive deletion of *pfhrp2*/3 genes in clinical isolates from Ethiopia. This was evident for *pfhrp*2 and its structural homolog *pfhrp*3 as well as their respective flanking genes. In order to rule out the possibility that this was caused by primers we used alternative primers with different binding sites and amplification conditions. In addition, we used another locus, *pfmdr*1, to rule out the possibility of low DNA quality being an issue. Furthermore, we used 18ssRNA to confirm microscopically determined *P. falciparum* positive specimens.

From all the six targets of *pfhrp*2/3 and flanking regions (Table 2), at least one locus was amplified for the 50 studied samples from a total of 64 confirmed *P. falciparum* clinical samples. In all 50 samples, *pfhrp*2/3 genes had deletions which continued to their flanking regions although deletions here were to a lesser degree. The lack of prior data on *pfhrp*2/3 deleted parasites in the country limits our investigation on whether these deletions are recent events or were present prior to the introduction of *pfhrp2*-based RDTs. For Peru, for instance, the appearance of these parasites was evident before the introduction of RDTs, which perhaps show the strength of selective forces on these deletions [7]. In contrast, a recent

mathematical modelling study showed that the use of *pfhrp*2-based RDTs is sufficient to select *P. falciparum* parasites lacking this protein.

Interestingly, in Ethiopia *pfhrp*2-based RDTs are far more popular than pLDH-based RDTs which could be because of their higher sensitivity for *P. falciparum* diagnosis. However, the fact that both *pfhrp2* and *pfhrp*3 deletions were detected in all of our samples probably indicate that this could have been a result of a recent selection. Furthermore, parasites lacking both genes are quite rare to find in other African countries, though this is quite prevalent in South American countries [7, 10]. Indeed, a recent whole genome study on *P. falciparum* isolates from 15 African countries has shown a highly divergent Ethiopian *P. falciparum* population, defining a genomic background that could likely dictate a different response to selective forces on the parasite [23]. A similar neighbouring parasite population in Eritrea also reported very high frequencies of these deletions [8, 9].

RDT-based treatment could be a determining factor to selectively clear non-deleted infections and hence increase the rate of spread of parasites with deletions [24]. However, the benefits of this mutation to the parasites and if RDT-guided treatments may have selected for *pfhrp2*-deleted mutants are yet to be determined. In Eritrea where malaria prevalence is quite similar to Ethiopia, *pfhrp2*-negative parasites had lower genetic diversity compared with that of *pfhrp2*-positive parasites and formed a closely related cluster probably caused by selection by use of *pfhrp*2-based RDTs [7]. Given that the observation of partial or complete deletion of the *pfhrp*2 gene in 2010 in South America sparkled the recommendation against the use of *pfhrp2*-based RDTs in these areas, the results here are relevant for future policy in Ethiopia [25–27]. This needs to take in consideration some limitations, such as the absence of data on diagnostics outcome for the samples based on RDTs that targets the *pfhrp2/3* genes. The fact that patient recruitment was not made using *pfhrp2*/3-based RDTs while the samples were collected makes it difficult to determine if the isolates would test negative and/or positive in the absence of *pfhrp*2/3 genes. Further studies using microscopy and *pfhrp2*/3-based RDTs are required at large scale to determine the extent of *pfhrp2*/3 gene deletions and the role the deletion could play in the test results in Ethiopia. The samples were also collected from one region (health centre) of the country, warranting future and extensive studies to further inform the national malaria control program (NMCP) on diagnostic approaches in Ethiopia as the elimination agenda in pursued. The routine use of RDTs may allow the parasites to escape detection by *pfhrp2*-based RDTs and may be selected to expand in the population. Worth noting is that although the *pfhrp2*/3 genes were deleted and/or absent, the respective flanking regions were amplified and they may contribute to reactivity of *pfhrp2*/3-based RDTs. Indeed, the sequence variation and deletion in *Pfhrp2/3* genes in Ethiopian isolates may not likely to negatively influence performance of currently used *pfhrp2* RDTs given that the RDTs targeting *hrp*2/3 genes are massively used in the area.

Conclusions

P. falciparum parasite populations with deletions of the *pfhrp2* and *pfhrp3* genes are present in Ethiopia and further large scale studies are required to identify the prevalence of deletion and its effect on current

RDTs-based diagnosis, towards adaptation of malaria control strategies.

Abbreviations

DNA
Deoxyribonucleic acid
GTS
Global Technical Strategy
Hrp2/3
histidine rich protein 2/3
NMCP
National malaria control program
*Pfhrp*2/3
Plasmodium falciparum histidine rich protein 2
PCR
Polymerase chain reaction
pLDH
Plasmodium lactate dehydrogenase
RDTs
rapid diagnostic tests
RNA
Ribonucleic acid
rRNA
18S ribosomal RNA
rDNA
18 S ribosomal DNA
WHO
World Health Organization

Declarations

Authors' contributions

LG collected the samples and drafted the manuscript. AM and AAN, analysed and interpreted the data. GS did the molecular test. All authors discussed the results and contributed to the final manuscript. All authors read and approved the final manuscript

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Competing interests

The authors declare that they have no competing interests

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Availability of data and materials

All data and materials supporting the results of the manuscript will be available at gote.swedberg@imbim.uu.se and lgolassa@gmail.com upon request

Ethics approval and consent to participate

Ethical approval for the study was granted by Aklilu Lemma Institute of Pathobiology, Addis Ababa University Institutional Review Board.

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28. **Additional file 3**. **Primer sequences for** pfhrp2, pfhrp3 **and their respective flanking genes**.

Supplementary Files

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