Molecular surveillance of *pfhrp2* and *pfhrp3* deletions in *Plasmodium falciparum* isolates from Mozambique

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**Abstract**

**Background:** Malaria programmes use *Plasmodium falciparum* histidine-rich protein-2 (PfHRP2) based rapid diagnostic tests (RDTs) for malaria diagnosis. The deletion of this target antigen could potentially lead to misdiagnosis, delayed treatment and continuation of active transmission.

**Methods:** *Plasmodium falciparum* isolates (n = 1162) collected in Southern Mozambique were assessed by RDTs, microscopy and/or 18SrRNA qPCR. *pfhrp2* and *pfhrp3* deletions were investigated in isolates from individuals who were negative by RDT but positive by microscopy and/or qPCR (n = 69) using gene-specific PCRs, with *kelch13* PCR as the parasite DNA control.

**Results:** Lack of *pfhrp2* PCR amplification was observed in one of the 69 isolates subjected to molecular analysis [1.45% (95% CI 0.3–7.8%)].

**Conclusions:** The low prevalence of *pfhrp2* deletions suggests that RDTs will detect the vast majority of the *P. falciparum* infections. Nevertheless, active surveillance for changing deletion frequencies is required.

**Keywords:** Malaria, Deletion, RDT, Mozambique, Pfhrp2

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**Background**

Malarial parasites exhibit striking genetic plasticity that allows their rapid adaption to new drugs [1] and detection methods [2, 3]. This adaptability of the parasite endangers preventive and therapeutic measures against malaria, as the success of control programmes largely relies on early diagnosis and effective treatment. Rapid diagnostic tests (RDTs) are commonly used in malaria case management and elimination programmes, particularly in remote areas where facilities for microscopy are not available [4].

*Plasmodium falciparum* histidine-rich protein-2 (PfHRP2), together with *Plasmodium* lactate dehydrogenase and aldolase, are the key target antigens in commercially available RDTs [5]. Evidence from South America, India and Africa [2, 3] suggest that “deletion” of the target epitope within the parasite PfHRP2 antigen could adversely impact the life of an affected individual as a consequence of delayed or no treatment. Besides *pfhrp2*, *pfhrp3* also affects the performance of RDT, as it has sequence homology with the *pfhrp2* and can be detected by the monoclonal antibodies used against PfHRP2 in RDTs [6].

With increasing false negative RDT reports in African countries, WHO has considered the need of rigorous monitoring of malaria parasites that lack the *pfhrp2* gene [2, 3, 7]. RDTs were introduced in Mozambique in 2007 and national wide use started in 2010 [8]. However, there is no information available about the extent of *pfhrp2*...
and pfhrp3 deletions in *P. falciparum* parasites circulating in Mozambique. In this context, this study aimed to assess the presence of pfhrp2 and pfhrp3 deletions in *P. falciparum* isolates from Manhiça and Magude districts of Southern Mozambique.

**Methods**

**Study site and design**

Between 2010 and 2016, a total of 9124 blood samples were collected onto filter papers during cross-sectional studies conducted at the beginning (November) or end (May) of the malaria season in Southern Mozambique (Manhiça and Magude; Table 1). In Mozambique, a peak in transmission is usually seen during the rainy season, from November to April. Transmission intensity in southern Mozambique is generally low, although areas of high transmission may still be observed [9].

Malaria diagnosis was conducted using microscopy, HRP2-based RDT and qPCR; or only RDTs and qPCRs. The inclusion criteria for the deletion analysis were: 1) a negative HRP2-based RDT (SD BIOLINE Malaria Antigen *P. f*—05FK50) but positive by microscopy and qPCR (18S rRNA) or 2) a negative HRP2-based RDT but positive qPCR (18S rRNA) if microscopy was not performed. First, a nested PCR targeting single copy *k13* gene (nPCRk13) was performed to verify the presence of parasite DNA in the sample [10]. Second, *pfhrp2* and *pfhrp3* genes were amplified using standard primers as described elsewhere [5, 11]. Finally, *pfhrp2* and *pfhrp3* deletions were concluded if *kelch13* gene PCR was positive, but PCRs for *pfhrp2* and *pfhrp3* failed to amplify the respective gene. The laboratory-adapted culture lines 3D7 as a positive control for both *pfhrp2* and *pfhrp3*, and HB3 and DD2 as negative controls for *pfhrp3* and *pfhrp2*, respectively, were amplified simultaneously. The National Mozambican Ethics Review Committee and the Hospital Clínic of Barcelona Ethics Review Committee approved the collection of samples and molecular analysis. Informed consent and permission (in the case of children under 18 years of age) were also obtained from each participant or a parent/legal guardian during the cross-sectional studies.

**Microscopy**

Thin and thick blood smears were air-dried, stained with Giemsa and examined using a light microscope fitted with a 100 × oil immersion lens and a 10 × eyepiece to quantify parasitaemia in the Centro de Investigação em Saúde de Manhiça (CISM) laboratory [9]. Slides were read twice by two different qualified microscopists, and if there was discordance in the results, a third reading was performed by an additional microscopist.

**Rapid diagnostic test**

A trained laboratory technician collected approximately 10 μL of blood from an individual by finger-prick to perform an RDT. The PFHRP2-based RDT (SD BIOLINE Malaria Antigen *P. f*—05FK50) was used as per the manufacturer’s instructions.

**DNA extraction and *Plasmodium falciparum* detection by real time PCR (qPCR)**

DNA was extracted from a half of the filter paper (Whatman, 903TM), containing a 25 μL blood drop by using QIAamp DNA Mini kit (Qiagen). The ABI PRISM 7500 HT Real-Time System (Applied Biosystems) was used to amplify purified parasite DNA templates, using a previously described method [12, 13]. Parasitaemia in the clinical samples was quantified by extrapolation against the standard curve prepared from an in vitro culture of 3D7 strain.

**kelch13 nested PCR (nPCRk13)**

Purified DNA templates were amplified using 2720 Thermal Cycler (Applied Biosystems) following a previously described method for the *kelch13* gene [10].

**Table 1  *P. falciparum* isolates collected during cross-sectionals with diagnostic results**

| Years | Place   | Samples collected | *P. falciparum* positive samples | RDT negative, microscopy and qPCR positives samples | RDT negative and only qPCR positives samples |
|-------|---------|-------------------|----------------------------------|-----------------------------------------------|----------------------------------------------|
| 2010  | Manhiça | 969               | 105                              | 1                                             | –                                            |
| 2011  | Manhiça | 842               | 138                              | 3                                             | –                                            |
| 2012  | Manhiça | 924               | 116                              | 3                                             | –                                            |
| 2013  | Manhiça | 829               | 166                              | 8                                             | –                                            |
| 2014  | Manhiça | 908               | 211                              | 7                                             | –                                            |
| 2015  | Manhiça | 770               | 93                               | 9                                             | –                                            |
| 2015  | Magude  | 981               | 101                              | 7                                             | –                                            |
| 2015  | Magude  | 1322              | 174                              | –                                             | 125                                          |
| 2016  | Magude  | 1579              | 58                               | 1                                             | –                                            |
pfhrp2 and pfhrp3 PCRs

Samples with intact parasite DNA confirmed by nPCR_{K13} were used for further amplification of region covering exon 1 and 2, as well as exon 2 of pfhrp2 and pfhrp3 genes [5, 11], following previously described methods with minor changes. These changes include the use of 1× HOT FirePol Master Mix, annealing temperatures of 63 °C of 1 min for across regions of exon 1 and 2 of pfhrp2 gene and 60 °C of 1 min for exon 2 amplification for both pfhrp2 and pfhrp3 genes. PCR products were visualized using 2% agarose (Invitrogen) and a UV trans-illuminator.

Results

Among the 9124 blood samples collected from participants in cross-sectional studies conducted in Southern Mozambique between 2010 and 2016, 1162 were P. falciparum positive by qPCR and/or by microscopy and RDTs. Among these 1162 P. falciparum isolates, 164 samples were found eligible for the pfhrp2 and pfhrp3 deletion analysis based on a RDT negative, microscopy positive and qPCR positive results (MO+/RDT−/qPCR+; n = 39), or an RDT negative and qPCR positive result (RDT−/qPCR+; n = 125). Filter papers and corresponding DNAs were available for 155 (95%) of these 164 P. falciparum isolates. Among these, 70 (45%) were positive by nPCR_{K13} (849 bp amplicon size; Fig. 1). Median qPCR parasite densities of the P. falciparum isolates that were negative by nPCR targeting kelch13 gene was 2.17 parasites/µL (interquartile range 1.2–4.4 parasites/µL).

69 samples were analysed for pfhrp2 and pfhrp3 deletions, as one DNA sample was not enough for the analysis (Fig. 1). Parasite densities in these samples ranged from the 3 to 330,214 parasites/µL by qPCR (Table 2). The laboratory 3D7 strain returned all the expected PCR products of pfhrp2 (exon 1–2 = 303 bp and exon 2 = 816 bp) and pfhrp3 (exon 1–2 = 301 bp and exon 2 = 719 bp). As expected, laboratory strains DD2 and HB3 lacked pfhrp2 and pfhrp3 amplifications respectively (Fig. 2).
Table 2  Parasite densities (parasites/µL of blood), age, sex and year of sample collection of the samples included in the study

| Years | Place     | Parasitemia by microscopy | Parasitemia by qPCR | Sex  | Age (in years) |
|-------|-----------|---------------------------|---------------------|------|---------------|
| 2010  | Manhiça   | 1670                      | 3858.5              | Male | 4             |
| 2013  | Manhiça   | 56                        | 33.5                | Male | 12            |
| 2013  | Manhiça   | 546                       | 600.3               | Male | 2             |
| 2013  | Manhiça   | 143603                    | 25250               | Male | 3             |
| 2013  | Manhiça   | 203                       | 1500                | Male | 14            |
| 2013  | Manhiça   | 39                        | 13.2                | Female | 24 |
| 2014  | Manhiça   | 232                       | 996.6               | Male | 4             |
| 2014  | Manhiça   | 44594                     | 23469.6             | Female | 15 |
| 2014  | Manhiça   | 140814                    | 84817               | Female | 3 |
| 2014  | Manhiça   | 5721                      | 3827.8              | Female | 7 |
| 2014  | Manhiça   | 52                        | 657.5               | Female | 3 |
| 2015  | Manhiça   | 386                       | 167.58659           | Female | 14 |
| 2015  | Manhiça   | 1657                      | 3407.6235           | Female | 8 |
| 2015  | Manhiça   | 100                       | 98.169426           | Female | 2 |
| 2015  | Manhiça   | 325                       | 156.13457           | Male | 2             |
| 2015  | Manhiça   | 51                        | 32.917961           | Male | 11            |
| 2015  | Manhiça   | 99                        | 108.99004           | Male | 17            |
| 2015  | Manhiça   | 5648                      | 1463.4641           | Female | NA |
| 2015  | Magude    | 3610                      | 1610.6819           | Female | 4 |
| 2015  | Magude    | 2950.5                    | 676.75568           | Male | 9             |
| 2015  | Magude    | 303.5                     | 224.56026           | Female | 50 |
| 2015  | Magude    | 928.5                     | 384.57782           | Male | 2             |
| 2015  | Magude    | 2637                      | 95.924171           | Female | 11 |
| 2015  | Magude    | NA                        | 14.8498             | Male | 7             |
| 2015  | Magude    | NA                        | 2.44336             | Female | 10 |
| 2015  | Magude    | NA                        | 27.025              | Male | NA            |
| 2015  | Magude    | NA                        | 2.70269             | Female | 15 |
| 2015  | Magude    | NA                        | 20.8876             | Female | 47 |
| 2015  | Magude    | NA                        | 330214              | Female | 3 |
| 2015  | Magude    | NA                        | 99.4996             | Female | 4 |
| 2015  | Magude    | NA                        | 14.9045             | Female | 12 |
| 2015  | Magude    | NA                        | 8.46764             | Female | 7 |
| 2015  | Magude    | NA                        | 9.3634              | Male | 3             |
| 2015  | Magude    | NA                        | 69.8859             | Male | 43            |
| 2015  | Magude    | NA                        | 4.21137             | Female | 17 |
| 2015  | Magude    | NA                        | 4.75359             | Female | 9 |
| 2015  | Magude    | NA                        | 314.461             | Male | 4             |
| 2015  | Magude    | NA                        | 104.915             | Male | 19            |
| 2015  | Magude    | NA                        | 25.1698             | Female | 11 |
| 2015  | Magude    | NA                        | 27.3208             | Male | 28            |
| 2015  | Magude    | NA                        | 20.9188             | Female | NA |
| 2015  | Magude    | NA                        | 1184.68             | Male | 15            |
| 2015  | Magude    | NA                        | 4.68228             | Female | 9 |
| 2015  | Magude    | NA                        | 612.026             | Female | 12 |
| 2015  | Magude    | NA                        | 182.307             | Female | 7 |
| 2015  | Magude    | NA                        | 61.8145             | Female | 5 |
| 2015  | Magude    | NA                        | 6.33165             | Female | 2 |
| 2015  | Magude    | NA                        | 50.2857             | Female | 40 |
No amplification was noticed in negative controls (with water and human genomic DNA), which confirms the *P. falciparum* specificity of all the primer sets used in this study. Expected PCR products were observed upon the amplification of regions across exon 1 and exon 2, as well as exon 2 of *pfhrp2* and *pfhrp3* genes in all the samples except one sample (H1.3). PCR amplification of region covering *pfhrp2* exon 1 and exon 2, as well as exon 2 was not observed in isolate H1.3 (Fig. 2a), while obtaining a positive amplification product for *kelch13* gene. This lack of amplification was confirmed in a second and independent PCR test. The microscopy and qPCR parasite density of sample H1.3 were 2950.5 and 676.75 parasites/µL, respectively. Upon case investigation, this sample was found to correspond to a 30 months old male child from Magude who reported previous episodes of fever (during last 30 days), lived in a fumigated household and slept under a bed net the night before the sample was collected. Apart from this, varying *pfhrp2* and *pfhrp3* exon 2 PCR products lengths (600–1000 bp) were also observed in the analysed samples.

**Discussion**

This study provides the first evidence of *pfhrp2* deletion in *P. falciparum* isolates circulating in Southern Mozambique. The prevalence of 1.45% (95% CI 0.3–7.8%) *pfhrp2* deletion among analyzed samples is low as compared to the prevalence observed in other malaria endemic countries such as India (2.4%), Senegal (2.4%), Mali (5%) and Ghana (30.3%) [14–17]. As per WHO guidelines, 5% prevalence of *pfhrp2* deletion has been considered as a minimum threshold to change the type of RDTs [3]. Therefore, PfHRP2-based RDTs are likely to detect the vast majority of the malaria parasites in southern Mozambique, but careful periodic monitoring for changes in deletion frequencies may be required to identify cases such as the single mutant detected in this study.

Previous reports have shown that *pfhrp3* deletion could be an early warning signal for *pfhrp2* deletion [11]. However, the *pfhrp3* deletion has not been observed in the present study. Since only blood spots on filter paper were available in this study, plasma PfHRP2 protein levels or RNA based assays for the same sample could not be performed. However, a number of independent *pfhrp2* PCR based investigation was done to confirm the lack of *pfhrp2* gene in the *P. falciparum* isolate. Moreover, as significant amount of *P. falciparum* isolates (n = 164) were detected by real time PCR but not by nested PCR, given lower sensitivity of the latter [18], and consequently were not eligible for *hrp2/hrp3* assessment. Finally, the varying length of exon 2 of *pfhrp2* and *pfhrp3* PCR products

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**Table 2 continued**

| Years | Place  | Parasitemia by microscopy | Parasitemia by qPCR | Sex | Age (in years) |
|-------|--------|---------------------------|---------------------|-----|---------------|
| 2015  | Magude | NA                        | 90.4941             | Male | 1             |
| 2015  | Magude | NA                        | 14.7296             | Male | 3             |
| 2015  | Magude | NA                        | 539.879             | Female | 35           |
| 2015  | Magude | NA                        | 8.85961             | Male | 12            |
| 2015  | Magude | NA                        | 231.339             | Female | 8            |
| 2015  | Magude | NA                        | 19.9863             | Male | 15            |
| 2015  | Magude | NA                        | 55.4191             | Female | 2            |
| 2015  | Magude | NA                        | 73.8065             | Male | 12            |
| 2015  | Magude | NA                        | 24.4031             | Female | 29           |
| 2015  | Magude | NA                        | 419.439             | Female | 27           |
| 2015  | Magude | NA                        | 158.95              | Male | 12            |
| 2015  | Magude | NA                        | 6.46944             | Female | 45           |
| 2015  | Magude | NA                        | 9.88753             | Female | 40           |
| 2015  | Magude | NA                        | 2.68125             | Male | 18            |
| 2015  | Magude | NA                        | 78.1784             | Female | 13           |
| 2015  | Magude | NA                        | 20.7993             | Male | 15            |
| 2015  | Magude | NA                        | 576.566             | Female | 11           |
| 2015  | Magude | NA                        | 54.7864             | Male | 9             |
| 2015  | Magude | NA                        | 19.1635             | Female | 35           |
| 2015  | Magude | NA                        | 491.664             | Male | 45            |
| 2016  | Magude | 609                       | 645.284             | Male | 31            |

NA not available
indicates the presence of different numbers of previously identified amino acid repeats [5].

According to 2016 WHO world malaria day fact sheet, the use of RDT has significantly increased globally from 46 million sold in 2008 to 314 million in 2014. In 2014, 53% of global RDTs (P. falciparum-specific tests) were delivered to African countries [3]. The excessive use of PfHRP2 based RDTs might enhance the selection of P. falciparum isolates with pfhrp2 deletion, especially in endemic areas where pfhrp2 deletion is present. Thus, it is important to monitor the presence of parasites with pfhrp2 and pfhrp3 deletions to avoid false negative results by RDT. Limitation of the study is that the sample’s material was not available for amplification of flanking genes of pfhrp2 and pfhrp3 genes.

Conclusions

The low prevalence of pfhrp2 deletions suggests that RDTs will detect the vast majority of the P. falciparum infections in Mozambique. However, active surveillance to detect increases in pfhrp2 deletion frequencies is required towards the common goal to eliminate malaria.

Authors’ contributions

HG carried out deletion PCRs analysis, interpretation of results and wrote the draft of this manuscript. GM, BG, PC, LN and WS participated in fieldwork, collected clinical and epidemiological data and laboratory analyses. AM, JC, FS, NRR, PA and PA participated in the study design, interpretation of results and writing of this article. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets analyzed in this study are available from the corresponding author on request.

Consent for publication

All authors have given their consent for publication.

Ethics approval and consent to participate

The National Mozambican Ethics Review Committee and the Hospital Clinic of Barcelona Ethics Review Committee approved the collection of samples and molecular analysis. Informed consent and permission (in the case of children under 18 years of age) were also obtained from each participant or a parent/legal guardian during the cross-sectional studies.

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