Identification of Plasmodium falciparum isolates lacking histidine-rich protein 2 and 3 in Eritrea

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\section{A B S T R A C T}

The histidine-rich protein 2 of Plasmodium falciparum is the most common malaria antigen targeted by rapid diagnostic tests for the specific diagnosis of P. falciparum. Recently, pfhrp2 gene deletions have been documented in P. falciparum isolates from South America and some multiple endemic countries in Africa and Asia. Parasites with such gene deletions can produce false negative diagnostic results using HRP2-based rapid diagnostic kits. In the present work, the prevalence of P. falciparum parasites lacking pfhrp2, pfhrp3, which produces a second P. falciparum antigen that is recognized by PfHRP2-based rapid diagnostic tests, and their flanking genes was evaluated in 135 P. falciparum isolates from Gash Barka region and in 9 isolates from Debub region, in Eritrea. In the analyzed samples, 56\% (81/144) of isolates were pfhrp2/pfhrp3 positive, while 9.7\% (14/144) showed deletion of exon 2 of pfhrp2 gene and 43\% (62/144) of isolates lacked the pfhrp3 gene. These results suggest that the pfhrp2 and pfhrp3 deletion phenomenon is present in a considerable proportion in the study areas, thus making the HRP2/3 based rapid diagnostic tests not completely reliable for malaria diagnosis in Eritrea.

\section{1. Introduction}

Malaria is a public health challenge world-wide, with an estimated 3.2 billion people at risk for infection in 95 countries. Notwithstanding there has been a 48\% reduction in malaria mortality rates globally since 2000. The World Health Organization (WHO) estimates that 214 mil-lion cases occurred in 2015 which led to approximately 438,000 deaths (WHO, 2015).

Despite Eritrea being one of the least developed countries in the world, according to the (World Bank/Eritrea, 2016) this country has made significant progress in public health, including a consistent de-cline in malaria morbidity and mortality since 1999. Improvement in controlling malaria has been principally achieved by widespread im-plementation of prevention and control measures, including the use of rapid diagnostic tests (RDTs) (MoH, 2010). Between 2000 and 2012, the overall malaria morbidity and mortality have been reduced by 74\% and by 83\%, respectively (WHO, 2014). In 2012, the Malaria Indicator and Prevalence Survey showed a low parasite prevalence of 1.4\% by RDT and 1.1\% by microscopy indicating that the country is striving to achieve pre-elimination of malaria (WHO Country Cooperation Strategy 2014–2016: Eritrea). However, in the last three years, incidence of malaria has risen in four regions (Anseba, Debub, Gash-Barka, and Semenawi Keih Bahri) of the country.

The immuno-based assay RDT is a diagnostic method that detects malaria antigen in a finger-prick blood sample. The three main groups of antigens detected by RDTs are: Histidine rich protein 2 (PfHRP2), produced by thromphoziotes and young gametocytes of P. falciparum only; Parasite Lactate Dehydrogenase enzyme (pLDH) (falciparum spe-cific, P. vivax specific or pan specific), and Aldolase pan-specific enzyme (WHO, 2000).

PfHRP-2 is a histidine and alanine-rich protein, characterized by a highly polymorphic repeat domain and represents the most common malaria antigen targeted by RDTs for the specific diagnosis of P. falci-parum (Howard et al., 1986).

Frequently, a second protein of P. falciparum, the PfHRP3 antigen (Wellems and Howard, 1986) is recognized by PfHRP2-based RDTs (Baker et al., 2010; Lee et al., 2006).

In 2010, Gamboa et al. (2010) first documented the deletion of pfhrp2 and pfhrp3 genes in P. falciparum field isolates from Peru and, so far, such deletions have been recorded in many other countries (Houzé...
et al., 2011; Kumar et al., 2012; Koita et al., 2012; Kumar et al., 2013; Wurtz et al., 2013; Deme et al., 2014; Atroosh et al., 2015; Li et al., 2015; Bharti et al., 2016; Amoah et al., 2016; Parr et al., 2016; Kozycki et al., 2017). Additionally, the deletion of the pfhrp2-flanking genes, MAL7P1.228 and MAL7P1.230, and the pfhrp3-flanking genes, MAL13P1.475 and MAL13P1.485, were also observed in field falciparum isolates (Akinyi et al., 2013; Murillo Solano et al., 2015; Dorado et al., 2016; Bharti et al., 2016; Abdallah et al., 2015).

The above studies further revealed that polymorphisms of the pfhrp2/3 genes can affect the performance of HRP2-based RDTs in term of sensitivity up to total test failure (false-negative) when testing iso-lates that have pfhrp2 and/or pfhrp3 deletions.

Recently, a survey aimed at monitoring the quality of RDT performance in Eritrea showed a high rate of false-negative results using PHTP-R2 detecting RDTs, recommending molecular investigation on the polymorphism of the pfhrp2 gene as a potential cause of the RDT failure in this country (Berhane et al., 2017). In the present work, we screened 144 P. falciparum isolates from two regions (Gash Barka and Debub) in Eritrea for the presence of pfhrp2 and pfhrp3 genes and their flanking genes to investigate the presence of pfhrp-deleted plasmodium isolates.

2. Material and methods

A total of 144 microscopically confirmed P. falciparum mono-infected blood samples were collected in two endemic regions in Eritrea between November 2013 and November 2014: 5 samples from Agordat and 130 from Barentu, in the Gash Barka region, and 9 samples from Medefera, in the Debub region. Ethical approval for sample collection was obtained from the Ethics Committee of the Eritrean Ministry of Health. The recruited patients were 51 women, with a median age of 22.9 years (range 4–70 years), and 93 men, with a median age of 27.3 years (range 3–70 years). Following informed consent of the patient or their guardian, a blood sample by finger prick was collected, blotted in triplicate on a filter paper, and air dried. Total DNA was extracted using PureLink Genomic DNA Kits (Invitrogen, according to the manufacturer’s instruction. P. falciparum infections were confirmed by Real-Time PCR method as previously described by Veron et al. (2009). All samples investigated in the present study were also successfully amplified and sequenced for three single-copy genes (PfK13, Pfmdr1, Pfcrt), molecular markers associated with antimalarial drug resistance, as described in Menegon et al., 2016.

In the present study, all samples were tested for exon 2 of pfhrp2 (Mal7P1.231) and pfhrp3 (Mal7P1.480) genes, by PCR amplification, using primers Pfhrp2-F1 (5′-AAAAGACTTAATTAAATAAGAG-3′)/Pfhrp2-R1 (5′-AATAATATTAGGCAGTGGCA-3′) and Pfhrp3-F1 (5′-AATGCAAAAGACTTAATTAC-3′), Pfhrp3-R1 (5′-TGTTGTAAGTGAGGTAGT-3′), respectively, previously designated by Baker et al. (2005). In addition, the extension of the deletion in pfhrp2 and pfhrp3-flanking genes was evaluated by PCR amplification of MAL7P1.228 (PF3D7–0831200), MAL7P1.230 (PF3D7–0831900), MAL13P1.475 (PF3D7–1372100) and MAL13P1.485 (PF3D71372300) genes using the outward primers described in Gamboa et al. (2010). In summary, PCR amplifications were done in a final volume of 20 μl containing 3 μl of DNA of each samples, 10 μl of EconoTaq® PLUS 2× Master (Lucigen Corporation WI, USA) and 0.5 μM of each primers.

Genomic DNA from Dd2 (pfhrp2−), HB3 (pfhrp3−) and 3D7 (wild type) were used as controls for the PCR amplifications. The PCR cycling parameters were as follows: 50 cycles of 94 °C for 50 s, 50 °C for 50 s, 72 °C for 1 min, for pfhrp2/pfhrp3 amplifications and 50 cycles of 94 °C for 50 s, 55/60 °C for 50 s, 72 °C for 1 min for the amplification of flanking genes. The PCR products were resolved on 2% ethidium bro-side stained agarose gels, with 0.5 μg/ml Gel Ready 100 bp DNA ladder (Lucigen) loaded on the same gel, and visualized under UV transilluminator.

3. Results

Of the total 144 isolates screened in the present work, 27 (18.7%) isolates were collected in 2013 in Gash Barka region, while a total of 117 (81.3%) were gathered in 2014, including all the 9 isolates from Debub region. Among 144 analyzed P. falciparum isolates, pfhrp2 and pfhrp3 PCR fragments were successfully amplified in slightly more than half of the isolates (56.2%), specifically in 78 isolates from Gash Barka region and 3 from Debub region (Fig. 1). Fourteen isolates (9.7%), of which 12 collected in 2014 in Gash Barka region, showed deletion of exon 2 of Pfhrp2, while a total of 62 isolates (43%) lacked the pfhrp3 gene. Among these isolates, complete lack of Pfhrp2/Pfhrp3 genes was identified in 13 isolates (9%), 11 from Gash Barka region; for all thirteen double-negative isolates a PCR amplification was repeated both for the two genes to confirm this result and, at the same time, for Pfmdr1 gene, as described by Wooden et al. (1993), to confirm the integrity of the parasite DNAs. Taking into account the characterization of the flanking genes, we observed a more frequent deletion of the gene MAL13P1.475 (PF3D7–1372100), flanking Pfhrp3 compared to hrp2-flanking genes. Overall, sixteen different pfhrp2 and pfhrp3 deletion patterns were observed in our samples (Table 1).

4. Discussion

Malaria RDTs are useful tools to confirm presence of malaria. Their use has increased in the last decades either for surveillance purposes especially in countries with underdeveloped health systems, or for malaria management or for diagnosis in travelers returning from tro- pical countries. Under optimal conditions, the sensitivity of the RDTs is considered similar to that of direct microscopy (Cheng et al., 2014). However, their execution under field conditions may be questionable since on a number of occasions false negative results have been en countered which would negatively affect proper early therapeutic
intervention. False negatives can be due to several causes, notably among these are: altered storage conditions and transportations, poor product quality, impairment in host and parasite density or antigen concentration. In any case, the reasons for the basis of RDT false negative results should be investigated in depth, in particular in cases related to parasites not expressing target antigens or expressing anti-variant variants. In fact, P. falciparum parasites not expressing PfHRP2 and/or PfHRP3 antigens have been reported both in laboratory strains and field isolates (Cheng et al., 2014).

Recently, pfhrp2 deletions have been documented in Mali in 2% of isolates, with a total estimated frequency of 10–15% (Koita et al., 2012) and in Senegal (2.4% of isolates) (Wurtz et al., 2013). In Ghana the percentage of isolates lacking the pfhrp2 gene is 22–40% (Amoah et al., 2016). Markedly high prevalence of pfhrp2-deleted mutants was also found in Democratic Republic of Congo (Parr et al., 2016) and in Rwanda (Kozycki et al., 2017). However, the phenomenon of deletions is not spread throughout Africa.

In a study carried out in Gambia, Kenya, Mozambique, Tanzania and Uganda, where parasite samples from patients admitted with a diagnosis of severe malaria but with very low PFHRP2 plasma levels have been analyzed, there was no evidence for deletion of either pfhrp2 or pfhrp3 genes (Ramutton et al., 2012).

In late September 2015, the National Malaria Control Program/CDC of Eritrea reported to the WHO high rate of false negative RDT results in microscopically confirmed P. falciparum cases. This initiates an investigation to explore the sensitivity of RDTs under field conditions (Berhane et al., 2017). The investigation revealed that HRP2 –target RDTs, in particular SD Bioline RDTs, under field condition returned 80% of false negative results in 50 microscopically confirmed P. falciparum infections.

The current work showed, firstly, the evidence for pfhrp2 and pfhrp3 deletions in P. falciparum parasite isolates collected from two regions in Eritrea.

Even if one limitation of the study is the lack of any information about the performance of RDTs on the processed samples, our results are consistent with those reported in the Berhane's study. In the Berhane's study 11 out of 15 analyzed samples from Gash Barga region (7 from Agordat and 8 from Barentu) failed to be recognized by RDTs, specifically 100% of samples from Agordat and 37.5% of samples from Barentu provided false negative results. Our results showed that 60% (3/5) of isolates from Agordat were pfhrp3-negative, and about 40.7% (53/130) of the isolates from Barentu were pfhrp2 and/or pfhrp3-negative.

In Mendefera (Debub region), 50% of samples were found to be RDT negative in the Berhane's study, which is in keeping with our finding of 66% rate of pfhrp2 and/or pfhrp3 deletions in isolates from this region. It would appear that the high prevalence of P. falciparum parasites lacking pfhrp3 exon 2 gene alone or in addition to pfhrp2 as evidenced in Gash Barka and Debub regions can be associated with RDT failures in these regions.

According to the recently released WHO guidelines for malaria control programs (WHO, http://apps.who.int/iris/handle/10665/208819) pfhrp2/3 deletions should be suspected when microscopy-positivity rates are systematically > 10–15% higher than RDT-positive rates and if pfhrp2/3 deletions are found to be prevalent. In such in-stances, an investigation of pfhrp2/3 deletions should be undertaken and diagnostic policies revisited.

In conclusion, due to the presence of parasites with pfhrp2-3 deletions in some Eritrean regions, the use of malaria RDT kits that can detect antigens other than PfHRP2/PfHRP3 such as the PfHRP2/ PfILDH combo RDT kit should be strongly encouraged, in order to en-hance malaria diagnosis in Eritrea.

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References


Table 1

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The symbol ‘+’ indicates gene amplification while ‘−’ indicates no gene amplification.

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WHO guidelines for malaria control programs http://apps.who.int/iris/handle/10665/208819.


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