First evidence of the deletion in the *Pfhrp2* and *Pfhrp3* genes in *P. falciparum* from Equatorial Guinea

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Abstract

Background WHO recommends RDTs as a good alternative malaria-diagnosis method in remote parts of sub-Saharan Africa. The majority of commercial RDTs currently available detect the *P. falciparum* protein histidine-rich protein 2 (PfHRP2). There have also been recent reports of Pfhrp2 deletions being found in parasites collected from several African countries. WHO has concluded that the lacking the Pfhrp2 gene must be monitored in Africa. The purpose of the study was to analyze why the samples that were positive by PCR were negative by RDTs; and, therefore, to determine whether there have been deletions in the Pfhrp2 and/or Pfhrp3 genes.

Methods Malaria NM-PCR was carried out to all the samples collected in the field. A group of 128 samples was positive by PCR but negatives by RDT, these samples were classified as RDT false-negatives. It was carried out a PCR for exon2 of Pfhrp2 and Pfhrp3 genes to detect the presence or absence of these two genes. Frequencies with 95% confidence intervals (CIs) were used for prevalence estimates. Associations were assessed by the chi-square test or Fisher's exact test. The level of significance was set at $p \leq 0.05$. Statistical analyses were performed using the software package SPSSv.15.0.

Results After the PCR, 81 samples were identified (4.7%, 95%CI: 3.8-5.8) which had deletion in both genes, Pfhrp2 and Pfhrp3 . Overall, however, 11 samples (0.6%, 95%CI: 0.36-1.14) had deletion only in Pfhrp2 but not in Pfhrp3 , and 15 (0.9%, 95%CI: 0.6-1.5) presented with deletion only in Pfhrp3 but not in Pfhrp2 . Considering the Pfhrp2 gene separately, within the total of 1,724 samples, 92 (5.3%, 95%CI: 4.37-6.5) had evidence of deletion.

Conclusion The present study provides the first evidence of deletion in the Pfhrp2 and Pfhrp3 genes in *P. falciparum* isolates from Equatorial Guinea. However, larger studies across different regions within the country and across different seasonal profiles are needed to determine the full extent of Pfhrp2 and Pfhrp3 deletion. It would be strongly recommendable to implement an active surveillance program in order to detect any increases in Pfhrp2 and Pfhrp3 deletion frequencies.

Background

Equatorial Guinea (EG) in Central West Africa is divided into two regions, the Insular Region (Bioko, Annobon) and the Continental Region (Rio Muni). Malaria remains a major public health problem in the country, and EG is a holoendemic area with year-round transmission [1]. According to official data from EG's National Malarial Control Program, the prevalence of *Plasmodium falciparum* malaria in the country (for children between 2 and 14 years old) was 12.5% in 2018. Malaria prevalence on Bioko Island was 10.3% and 46.5% in the Continental Region. The 2018 Malaria Report does not report prevalence for the various different species; however, 2011 data for the Continental Region shows that 95.2% of malaria infections were *P. falciparum* (Pf) and 9.5% *P. vivax* (Pv), with eight cases of mixed infection [2].

The main malaria-control strategy is quick and accurate diagnosis followed by effective treatment [3]. Early and accurate diagnoses are essential for both effective disease management and for proper malaria surveillance. The quality of malaria diagnosis is important in all settings, as misdiagnosis can result in significant morbidity and mortality. Since 2010, the WHO has recommended that all patients with suspected malaria should have their diagnoses confirmed by microscopy or a rapid diagnostic test (RDT) before treatment [4]. Microscopy and RDTs are the primary choices for malaria diagnosis in the field. Furthermore, in remote parts of sub-Saharan Africa, RDTs have become the primary tool for the parasitological diagnosis or malaria confirmation [5]. In the absence of well-trained technicians for microscopic diagnosis in many areas, the WHO recommends RDTs as a good alternative malaria-diagnosis method [6, 7].

RDTs are commonly used in malaria case-management and elimination programmes especially in remotes areas where microscopy facilities are not available [8]. As the tests are easy to perform and provide rapid results (15-20 min), they are exceedingly useful for rapid and malaria diagnosis in most malaria-endemic areas [9]. The majority of commercial RDTs currently available detect the *P. falciparum* protein histidine-rich protein 2 (PfHRP2) which *P. falciparum* only expresses in blood during the ring stage [10]. However, the failure to detect and treat false-negative infection increases the risk that people in a given community can contribute to onward infection through mosquitoes. .

The antibodies on the test strip detect the PfHRP2 antigen but may cross-react with proteins expressed by another member of the HRP gene family, PfHRP3, as there are very similar amino acid sequences [9]. Procurement decision-makers' general preference for PfHRP2-based RDTs is largely based on the findings of a number of studies which report that these tests are both more sensitive and heat-stable than the RDTs that detect other malaria antigens such as plasmodium lactate dehydrogenase, whether pan-pLDH (all species) or *P. falciparum*-specific (Pf-pLDH) or aldolase-based tests [11]. The major drawbacks for RDTs are false positives, because PfHRP2 persists in the blood for several days after an infection has been cleared [12]; and false negatives that can be due to *Pfhrp2/Pfhrp3* gene deletions, which has been observed for HRP2 in African field-isolates [13]. In 2010, Gamboa et al reported the first confirmed identification of *P. falciparum* parasites with *Pfhrp2/Pfhrp3* gene deletions; these parasites which expressed neither PfHRP2 nor PfHRP3 were identified in the Peruvian part of the Amazon River basin [9]. There have also been recent reports of HRP2 deletions being found in parasites collected from several African countries, including the Democratic Republic of the Congo, Ghana, Kenya, and Rwanda, in addition to in India [14-18]. Importantly, patients with false negatives may not receive treatment at all or may receive it later. Due to the increase in reports of RDT false-negatives in African countries, the WHO has concluded that malaria parasites lacking the *Pfhrp2* gene must be rigorously monitored [8].

RDTs were introduced in EG in 2010, although microscopy is still considered as the “gold standard” for malaria diagnosis in the country. In 2017, 60,798 RDTs were distributed in EG to different hospitals and Health Centers [19].

The objective of the present study was to analyze why the samples were positive by Nested Multiplex-PCR (NM-PCR) and microscopy were negative using RDTs; and, therefore, to determine whether there have been deletions in the *Pfhrp2* and/or *Pfhrp3* genes that could lead to false negatives.

Methods

Study area

The survey was carried out in the district of Bata in the Litoral Province of the Continental Region of EG, located between Cameroon and Gabon (Figure 1). The region has a tropical climate with two dry seasons (December to March and June to September) alternating with two rainy seasons (March to June and September to December). The mean daily maximum temperatures are 29-32°C and the minimum temperatures, 19-22°C.

Study population

The samples were collected from a cross-sectional survey conducted in June-August 2013 in Bata as part of a project called "PREVAMAL". A total of 1,741 individuals (1,043 in urban settings and 698 in rural) were recruited [20, 21]. Figure 1 shows the different locations where the samples were collected.

Blood samples were taken from participants' fingers for malaria diagnosis using both RDTs and microscopy. The blood was spotted on Whatman 903™ paper (GE Healthcare Bio-Sciences Corp.) for further molecular studies. The blood on the filter paper was air dried, stored in double zip-lock plastic bags with silica gel at 4°C, and subsequently transported to the National Center for Tropical Medicine, Institute of Health Carlos III, Madrid (Spain) for diagnostic confirmation by PCR.

Microscopy

The samples were taken in participants' homes, and the thick and thin slides were also prepared on site. The peripheral blood specimen slides were made immediately after collection on clean, grease-free microscope slides and allowed to air dry. The films were stained with 10% Giemsa solution (Applichem, Panreac ITW Companies) for 10 minutes, and examined by WHO-certified microscopists from the National Malaria Programme of Equatorial Guinea's Ministry of Health and Social Welfare. After air drying, each slide was subjected to an oil immersion objective lens examination all fields were examined before declaring a slide negative. For each specimen, the thick films were examined first in order to detect malaria parasites; the thin films of each specimen were then only examined for speciation in those instances when parasites had already been identified in the thick film. The slides were each examined by two microscopists; each specimen was examined independently, and the result was recorded as positive when both microscopists found both evidence of a malaria parasite, and identified the same species. In the event of a discrepancy, a third microscopist also assessed the slide.

Rapid diagnostic test

The RDT used *in situ* was the NADAL[®] Malaria 4 species test (Test cassette) (Nal von Minden, Moers, Germany). This test enables differential diagnosis between *P. falciparum* (Pf), *Plasmodium malariae* (Pm), *P. vivax* (Pv), and *P. ovale* (Po) in human whole-blood samples. It detects HRP2-specific proteins for Pf, and pLDH-specific proteins for Pf, Pv, Pm, and Po. The test has a sensitivity of 99.7% for Pf and 95.5% for non-Pf parasites with the microscopic detail of a large droplet, and a specificity of 99.5%. The cut-off level was 1 to 50 parasites/ml of blood for HRP2 and 51 to 100 parasites/ml of blood for pLDH. To perform the malaria test, 5 ml of whole blood is collected with the provided capillary pipette and transferred to the sample well. Four drops of the assay diluent are then added to the diluent well, in accordance with the manufacturer's protocol. The results are read after 15-20 min, only tests containing the control band are considered valid. Participants whose RDTs produced positive results were immediately offered treatment as set out by the EG national guidelines [22].

DNA extraction and molecular analysis

The DNA was extracted from the filter paper samples using commercial kits (Speedtools tissue DNA Extraction Kit, Biotools, Spain). It was used a 5 mm diameter punch that contained 10ml of blood.

1. **NM-PCR for the diagnosis of malaria:** the target selected in this NM-PCR is the gene encoding the 18S small subunit ribosome RNA (ssrDNA) and includes an internal amplification control to avoid false negatives (18S human rRNA) [23-25]. This was carried out on all the samples collected in the field, including both positives and negatives by microscopy and by RDTs irrespective of the result. After the NM-PCR tests had been completed, a group of 128 samples was identified for further study; these samples were positive using PCR and by microscopy, but negative using RDTs. Therefore, these samples were classified as RDT false-negatives.
2. **Nested PCR for *Pfdhfr*, *Pfdhps*, *Pfmdr1* and *Pfcrt* genes:** These genes were studied in the RDT false-negative samples, in accordance with the Maryland University protocols [26]. The nested-PCR included the following fragments of each gene: *Pfdhfr* (108/164, 51/59), in *Pfdhps* (400 and 500), in *Pfmdr1* (86/1246) and *Pfcrt* (76). This nested-PCR was used as a control for the quality of the DNA. Thus, if all the samples worked in the PCR of these genes, this indicates that the DNA has quality for the PCR. Therefore, if no exon2 amplification fragment is obtained from the *Pfhrp2* and *Pfhrp3* genes, it was not due to poor DNA quality or other factors, but because there was a true deletion in the *Pfhrp2/3*.
3. **PCR for exon2 of *Pfhrp2* and *Pfhrp3* genes:** the 128 samples with intact parasite-DNA confirmed by NM-PCR and *pfdhfr*, *pfdhps*, *pfmdr1* and *pfcrt* nested-PCR were used for further amplification of the exon 2 of *Pfhrp2* and *Pfhrp3*. This was to detect the presence or absence of these two genes [5, 6 and 27]; this test was performed as described previously, with the same primers but with some minor changes.

Pfhrp2-F1 (5'-CAAAAGGACTTAATTTAAATAAGAG-3') / **Pfhrp2-R1** (5'-AATAAATTTAATGGCGTAGGCA-3') were designed to anneal to the 5' and 3'ends of exon 2 of *Pfhrp2*. Semnested amplification was performed by use of the primers **Pfhrp2-F2** (5'-ATTATTACACGAACTCAAGCAC-3') and **Pfhrp2-R1**. The

same procedures and conditions were used to amplify the *Pfhrp3* gene by use of the primers **Pfhrp3-F1** (5'-AATGCAAAAGGACTTAATTC-3'), **Pfhrp3-R1** (5'-TGGTGTAAGTGATGCGTAGT-3'), and **Pfhrp3-F2** (5'-AAATAAGAGATTATTACACGAAAG-3'). These changes included the use of Biotools Hotsplit DNA polymerase (5U/ml) (Biotools B&M Labs, S.A. Madrid, Spain), and the PCR conditions were, 1st PCR y 2nd PCR: 95C for 15 min, followed 30 cycles by 95C for 1m, 60C for 1m, and 72C for 1 min and final extension 72C 10min, for both genes. *P. falciparum* 3D7 strain parasite was used as a positive control for *Pfhrp2* and Dd2 as a negative. This is because 3D7 is known to have all *Pfhrp2* and *Pfhrp3* genes, as well as the relevant flanking genes, while Dd2 lacks both *Pfhrp2* and its flanking genes. All the positive amplifications of *Pfhrp2* and *Pfhrp3* genes (exon2) were sequenced from both directions using forward and reverse primers of exon2. PCR products were purified with Illustra exoprostar 1-step (GE Healthcare Life Sciences) in accordance with the manufacturer's instructions and were then used in a standard dye terminator (Big Dye Terminator v3.1 Cycle Sequencing kit); the DNA was then sequenced using an ABI PRISM 3730 XL Analyser. BLAST (Basic Alignment Search Tool) was used for the sequence analysis, and homology with *Pfhrp2* and *Pfhrp3* of *P. falciparum* were established using MultAlin [28] and Sequence Manipulation Suite [29].

Statistical analysis

Frequencies with 95% confidence intervals (CIs) were used for prevalence estimates. Associations were assessed by the chi-square test or Fisher's exact test. The level of significance was set at $p \leq 0.05$. Statistical analyses were performed using the software package SPSSv.15.0.

Results

A total of 1,724 blood samples were diagnosed by microscopy, NM-PCR and RDT [21]. The *Plasmodium* sp. samples marked as negative by both microscopy and RDT were tested by NM-PCR as a quality control of the diagnoses. In this group of negative-by-RDT samples (n=963), 128 (7.4%) were identified as false negatives by PCR and were diagnosed as: 122 *P. falciparum*, 1 *P. falciparum*/*P. vivax*, 1 *P. malariae*, 1 *P. vivax* and 1 *P. ovale*. Figure 2 shows how the 1,724 samples were processed. These 128 negative by RDT samples could have been due to a number of possible causes: deletion in the *Pfhrp2* or *Pfhrp3* genes, a technicians' misinterpretation, or that there is a parasitemia in the sample that is lower than the minimum detection threshold for RDTs..

The DNA from the 128 false negatives by RDT were amplified correctly by the nested-PCR for the following genes of *P. falciparum* (Figure 3): *Pfdhfr* (108/164 and 51/59, product sizes 254bp and 113bp, respectively), *Pfdhps* (400 and 500, product sizes 148bp and 201bp, respectively), *Pfmdr1* (86 and 1246, product sizes 203bp and 295bp, respectively) and *Pfcrt* (76, product size 145bp). This indicates that the DNA has been extracted correctly, that it has no inhibition factors for PCR and that it has sufficient concentration to be used successfully in the PCR of exon2 of *Pfhrp2* and *Pfhrp3* genes. All the DNA samples were studied for the deletion of *Pfhrp2* and *Pfhrp3* using PCR (PCR for exon2 of the *pfhrp2* and *Pfhrp3* genes) the sizes of the expected fragments, if amplification did occur, were +/- 814bp for *Pfhrp2*

and +/- 719bp for *Pfhrp3*; this determined the presence or absence of these genes in the samples, Figure 4 shows the result of the PCR tests. The decision was made to perform the PCR for exon2 of *Pfhrp2* / *3* in all samples that were negative for RDT and positive for NM-PCR, although in some samples were diagnosed as non-*falciparum*. In the non-*falciparum* samples amplification fragment for *Pfhrp2* / *3* was not to be obtained. In this way it was also test the specificity of the PCR that is only valid for Pf, not giving false positives, that is, the non-*falciparum* samples acted as negative controls.

After the carrying out the PCR on the 128 RDT false-negative samples, the 5 non- *falciparum* (1 Pm, 3 Po and 1 Pv) samples were, as expected, negative in the *Pfhrp2*/*Pfhrp3*-PCR. The mix sample (Pf/Pv) were negative in the PCR for these two genes, therefore, was detected deletion in these genes for Pf. In the remaining 122 samples which were actually Pf, 81 samples were identified (4.7%, 95%CI: 3.8-5.8) out of 1,724 which had deletion in both genes (Table 1); therefore, the amplification fragment was absent; 15 samples (0.87%, 95%CI: 0.53-1.43) had no identifiable deletion in any of the genes studied. In this last case, the expected amplification fragments appeared, and were purified and sequenced, and after the comparison in BLAST they were found to have homology with the exon2 of the two genes under study. Overall, however, 11 samples (0.6%, 95%CI: 0.36-1.14) had deletion only in *Pfhrp2* but not in *Pfhrp3*, and 15 (0.9%, 95%CI: 0.6-1.5) presented with deletion only in *Pfhrp3* but not in *Pfhrp2*. Considering the *Pfhrp2* gene separately (the RDT detects the protein *Pfhrp2*), within the total of 1,724 samples, 92 (5.3%, 95%CI: 4.37-6.5) had evidence of deletion. In the mixed infection (*P. falciparum*/*P. vivax*) according to NM-PCR, neither the *Pfhrp2* nor the *Pfhrp3* genes were detected.

If the prevalence of deletion is calculated taking account the number of Pf detected by SnM-PCR (763) the frequencies for each case were: deletion in both genes, 10.6% (95%CI: 8.62-13); no deletion in any gene, 2% (1.97%; 95%CI: 1.19-3.22); deletion in *Pfhrp2* but not in *Pfhrp3*, 1.4% (95%CI: 0.81-2.56); deletion in *Pfhrp3* but not in *Pfhrp2*, 2% (1.9%; 95%CI: 1.19-3.22). Deletion just in *Pfhrp2* was 12% (95%CI: 9.94-14.56) (Table 1)

Discussion

This study provides the first evidence of *Pfhrp2* and *Pfhrp3* deletions in *P. falciparum* in Equatorial Guinea. The *Pfhrp2* deletion prevalence found in our samples was 5.3%; this prevalence is low when compared to that of Ghana (30%), but is very similar to Mali (5%) [30-32]. As yet, there is no data available for *Pfhrp2* and *Pfhrp3* deletion in neighboring Cameroon and Gabon. WHO guidelines consider a *Pfhrp2* deletion prevalence of 5% as a minimum threshold to change RDT types [33], or and if not possible, confirm the result of the RDT by another technique such as microcopy. This study identified 5.3% *Pfhrp2* deletion in the regional sample; moreover, this shows that it is now necessary to monitor the deletion of this gene across the whole country in order to obtain a complete picture of the deletions occurring with these genes. It is important to remember that this study was carried out in a district of Equatorial Guinea's continental region; therefore, it is evident that the study needs to be extended to cover the country in its entirety.

Deletions in *Pfhrp3* were also detected in the study, although the RDT used in EG and for this study was not designed to detect *Pfhrp3* proteins. In most settings, genetic mutations like *Pfhrp2/Pfhrp3* deletion in parasites are unlikely to be the main cause of RDT false-negatives unlike in this study and more studies are required to establish the true prevalence of these mutations in EG. In fact, there were some samples that were RDT false-negatives which were found to positive using NM-PCR, but without any detectable deletion in the *Phrp2* and *Pfhrp3* genes. Therefore, these results might be due to problems with the RDT used itself, or as result of operator errors when carrying out the tests and/or the interpretation RDT results; all of these could result in false-negatives [34].

Attributing false-negatives to *Pfhrp2/Pfhrp3* deletion has significant implications for public health policy. Once it has been established that the threshold has been passed, alternative RDTs will have to be procured and case management decisions will have to be revised, with re-training in the use of the new RDTs. Therefore, investigation into such deletions must be carried out systematically and accurately [7]. If *Pfhrp2* deletions are found to be prevalent among symptomatic individuals (the lower 95% confidence interval is still above 5%) as is the case, for example, in Eritrea and several countries in South America (Brazil, Colombia, Peru) national malaria control programs will have to switch to RDTs that do not exclusively rely on PfHRP2 to detect *P. falciparum*. A 5% threshold was selected by the WHO because it is somewhere around this point that the proportion of cases missed by PfHRP2 RDTs due to non-*hrp2* expression is likely to be greater than that which would be missed by using less-sensitive pLDH-based RDTs. A recommendation to switch can be further informed by mathematical modelling which shows whether parasites lacking PfHRP2 genes will spread under PfHRP2-only RDT pressure; policy makers may also decide to switch because of the complexity of procuring multiple RDTs and training staff in their use. In general, any change should be applied nationwide, although roll-out might be prioritized on the basis of PfHRP2-deletion prevalence in a given region [35]. Where microscopy is available, services should be strengthened to ensure that parasitological confirmation continues during the transition to new RDTs, and in order to investigate new suspected PfHRP2/PfHRP3 deleted parasite foci.

Excessive use of *Pfhrp*-based RDTs might enhance the selection of *P. falciparum* isolates with *Pfhrp2* deletion, especially in endemic areas where *Pfhrp2* deletion is present, as the case in Equatorial Guinea. Previous reports have also shown that *Pfhrp3* deletion can be an early warning sign for *Pfhrp2* deletion. Thus, it is important to monitor the presence of parasites with *Pfhrp2* deletions to avoid RDT false-negatives as well as *Pfhrp3* deletions to act as an early warning which offers public health bodies an opportunity to step up monitoring efforts and consider longer-term contingency plans [8, 9].

Conclusion

The RDTs used in this study detected the majority of *P. falciparum* infections as well as those from other species. Regarding with the deletion of the genes, it would be strongly recommendable to implement an active surveillance program in order to detect any increases in *Pfhrp2* and *Pfhrp3* deletion frequencies. Although there are false negatives due to causes other than deletion of these genes, a surveillance program is critical due to the level of frequencies of the deletion detected in the study. This surveillance

could be implemented through different regions and different seasonal profiles, needed to determine the full extent of *Pfhrp2* and *Pfhrp3* deletion.

To be able to control malaria, it is essential to have good diagnostic tools on the front line. To this end, the present study provides the first evidence of deletion in the *Pfhrp2* and *Pfhrp3* genes in *P. falciparum* isolates from Equatorial Guinea. If the frequency of deletion increases over time in the country, it might be important to think about changing the type of RDTs used.

Abbreviations

RDTs: Rapid Diagnostic Tests

HRP: histidine rich protein

CI: Confidence Interval

EG: Equatorial Guinea

PCR: Polymerase Chain Reaction

NM-PCR: Nested Multiplex PCR

N-PCR: Nested PCR

Phrp2: *Plasmodium falciparum* histidine rich protein 2

Phrp3: *Plasmodium falciparum* histidine rich protein 3

Pf-pLDH: *Plasmodium falciparum* lactate dehydrogenase

Pfdhfr: *Plasmodium falciparum* dihydrofolate reductase gene

Pfdhps: *Plasmodium falciparum* dihydropteroate synthase gene

Pfmdr1: *Plasmodium falciparum* multidrug resistant 1 gene

Pfcr1: *Plasmodium falciparum* chloroquine resistance transporter gene

Declarations

Ethics approval and consent to participate

The study was approved by the Minister of Health and Social Welfare of Equatorial Guinea (MINSABS) and the Ethics Committee of the Spanish National Health Institute, Carlos III (CEI PI 22_2013-v3). Written

informed consent for participation in the study was obtained from the caregivers interviewed and from the heads of the households.

Consent for publication

Not applicable

Availability of data and material

Not applicable

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

PB: is the corresponding author; conceived the original idea, carried out the molecular studies, and the writing of this article. All authors read and approved the final manuscript. **VG:** contributed to molecular studies. **LT:** contributed to the standardization of PCRs. **AM:** contributed to review the draft version. **MRB:** conducted the field study design, contributed sampling and review the draft version. **LG:** reviewed the manuscript. **PN:** contributed sampling, field work coordination. **MR:** provided support from the National Malaria Control Plan (Equatorial Guinea). **AB:** provided support from the National Centre of Tropical Medicine for the fieldwork and for the writing of this article.

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References

- [1] Benito A, Roche J, Molina R, Amela C, Alvar J. Application and evaluation of QBC malaria diagnosis in a holoendemic area. *Appl Parasitol.* 1994;35(4):266-72.
- [2] Mendes C, Dias F, Figueiredo J, Mora VG, Cano J, de Sousa B, et al. Duffy negative antigen is no longer a barrier to *Plasmodium vivax* molecular evidences from the African West Coast (Angola and Equatorial Guinea). *PLoS Negl Trop Dis.* 2011;5(6):e1192.
- [3] Gerstl S, Dunkley S, Mukhtar A, De Smet M, Baker S, Maikere J. Assessment of two malaria rapid diagnostic tests in children under five years of age, with follow-up of false-positive pLDH test results, in a hyperendemic falciparum malaria area, Sierra Leone. *Malar J.* 2010; 21;9:28.
- [4] WHO. Guidelines for the treatment of malaria: Geneve: World Health Organization, 2010. <http://apps.who.int/iris/bitstream/>.
- [5] Christina T. Kozycki, Noella Umulisa, Stephen Rulisa, Emil I. Mwikarago, Jean Pierre Musabyimana, 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.
- [6] Ugah UI, Alo MN, Owolabi JO, Okata-Nwali OD, Ekejindu IM, et al. Evaluation of the utility value of three diagnostic methods in the detection of malaria parasites in endemic area. *Malar J.* 2017;16(1):189.
- [7] Kim SH, Nam MH, Roh KH, Park HC, Nam DH. Evaluation of a rapid diagnostic test specific for *Plasmodium vivax*. *Trop Med Int Health.* 2008;13:1495–500.
- [8] Gupta H, Matambisso G, Galatas B, Cisteró P, Nhamussua L, Simone W, Cunningham J, Rabinovich NR, Alonso P, Saute F, Aide P, Mayor A. Correction to: Molecular surveillance of pfhrp2 and pfhrp3 deletions in Plasmodium falciparum isolates from Mozambique. 2017 Nov 14;16(1):464.
- [9] Gamboa D, Ho MF, Bendezu J, Torres K, Chiodoni PL, Barnwell J et al. A large proportion of *P. falciparum* isolates in the Amazon region of Peru lack *pfhrp2* and *pfhrp3*: implications for malaria rapid diagnostic tests. *PLoS One.* 2010;5:e8091.
- [10] Kumar Bharti P, Singh Chandel H, Krishna S, Nema S, Ahmad A, Udhayakumar V, Singh N. Sequence variation in Plasmodium falciparum Histidine Rich Proteins 2 and 3 in Indian isolates: Implications for Malaria Rapid Diagnostic Test Performance. *Sci Rep.* 2017 May 2;7(1):1308
- [11] Global Malaria Programme (Mayo 2016 revision september 2017)
- [12] Humar A, Ohrt C., Harrington MA, Pillai D. and Kain KC. Parasight test compared with the polymerase chain reaction and microscopy for the diagnosis of *Plasmodium falciparum* malaria in travelers. *Am. J. Trop. Med. Hyg.* 1997;56, 44-48.

- [13] Berhane A, Mihreteab S, Mohammed S, Embaye G, Hagos F, Zehaie A et al. PfHRP2 Detecting malaria RDTs: alarming false negative results in Eritrea 2016. American Society of Tropical Medicine and Hygiene 65th Annual Conference. ASTMH 2016;95(Suppl.5):Poster 879.
- [14] Parr JB, Verity R, Doctor SM, Janko M, Carey-Ewend K, Turmanet BJ et al. pfhrp2-deleted *Plasmodium falciparum* parasites in the Democratic Republic of the Congo: a national cross-sectional survey. J Infect Dis. 2017;216:36–44.
- [15] Amoah LE, Abankwa J, Oppong A. Plasmodium falciparum histidine rich protein-2 diversity and the implications for PfHRP 2-based malaria rapid diagnostic tests in Ghana. Malar J. 2016;15:101.
- [16] Beshir KB, Sep. Iveda N, Bharmal J, Robinson A, Mwanguzi J, Obukosia Busula A et al. Plasmodium falciparum parasites with 1 histidine-rich protein 2 (PfHRP2) and PfHRP3 gene deletions in two endemic regions of Kenya (submitted for publication).
- [17] Kozycki CT, Umulisa N, Rulisa S, Mwikarago EI, Musabyimana JP, Habima 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.
- [18] 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.
- [19] World malaria report. ISBN 978-92-4-156565-3. © World Health Organization 2018
- [20] Romay-Barja M, Jarrin I, Ncogo P, Nseng G, Sagrado MJ, Santana-Morales MA, et al. Correction: Rural-Urban Differences in Household Treatment-Seeking Behaviour for Suspected Malaria in Children at Bata District, Equatorial Guinea. PLoS One. 2015;10 (9): e0138518.
- [21] Berzosa P, de Lucio A, Romay-Barja M, Herrador Z, González V^{3,4}, García L, Fernández-Martínez A, Santana-Morales M, Ncogo P, Valladares B, Riloha M, Benito A. Comparison of three diagnostic methods (microscopy, RDT, and PCR) for the detection of malaria parasites in representative samples from Equatorial Guinea. Malar J. 2018 Sep 17;17(1):333.
- [22] Ncogo P, Herrador Z, Romay-Barja M, García-Carrasco E, Nseng G, Berzosa P et al. Malaria prevalence in Bata district, Equatorial Guinea: a cross-sectional study. Malar J. 2015;14:45.
- [23] Ta TH, Hisam S, Lanza M, Jiram AI, Ismail N, Rubio JM. First case of a naturally acquired human infection with *Plasmodium cynomolgi*. Malar J. 2014;24;13: 68.
- [24] Rubio JM, Post RJ, van Leeuwen WM, Henry MC, Lindergard G, Hommel M. Alternative polymerase chain reaction method to identify Plasmodium species in human blood samples: the semi-nested multiplex malaria PCR (SnM-PCR). Trans R Soc Trop Med Hyg. 2002; 96(1):S199–S204.

- [25] Ta TH, Ali-Tammam M, Lanza M, Rubio JM. Detection and identification of *Plasmodium* species by nested multiplex PCR. In manual of molecular methods for microbiological studies. 1st edition. Edited by Merino L, Giusa.
- [26] <http://www.medschool.umaryland.edu/malaria/Protocols/>
- [27] Baker J, McCarthy J, Gatton M, Kyle DE, Belizario V, Luchavez J, Bell D, Cheng Q. Genetic diversity of *Plasmodium falciparum* histidine-rich protein 2 (PfHRP2) and its effect on the performance of PfHRP2-based rapid diagnostic tests. J Infect Dis. 2005 Sep 1;192(5):870-7. Epub 2005 Jul 21.
- [28] Corpet F et al. Multiple sequence alignment with hierarchical clustering. 1998, Nucl. Acids. Res., 16(22), 10881-10890. (www.multalin.toulouse.inra.fr/multalin/)
- [29] www.bioinformatics.org/sms2/.
- [30] Amoah LE, Abankwa J, Oppong A. *Plasmodium falciparum* histidine rich protein-2 diversity and the implications for PfHRP 2: based malaria rapid diagnostic tests in Ghana. Malar J. 2016;15:101.
- [31] Koita OA, Doumbo OK, Ouattara A, Tall LK, Konaré A, Diakité M, et al. False-negative rapid diagnostic tests for malaria and deletion of the histidine-rich repeat region of the hrp2 gene. Am J Trop Med Hyg. 2012;86:194–8.
- [32] Ewurama D. A. Owusu, Samson K. Djonor, Charles A. Brown, Martin P. Grobusch, and Petra F. Mens. *Plasmodium falciparum* diagnostic tools in HIV-positive under-5-year-olds in two ART clinics in Ghana: are there missed infections? Malar J. 2018; 17: 92.
- [33] WHO. *Plasmodium falciparum* hrp2/3 gene deletions. Malaria Policy Advisory Committee Meeting. Geneva: World Health Organization; 2016. <http://www.who.int/malaria/mpac/mpac-sept2016-hrp2-consultation-short-report-session7.pdf>.
- [34] Kahama-Maró J, D'Acremont V, Mtasiwa D, Genton B, Lengeler C. Low quality of routine microscopy for malaria at different levels of the health system in Dar es Salaam. Malar J 2011;10:332.
- [35] Global Malaria Programme. False-negative RDT results and implications of new reports of *P. falciparum* histidine-rich protein 2/3 gene deletions. May 2016 (Rev September 2017). World Health Organization.

Table

Table 1.- Samples in which deletion is detected in *Pfhrp2* and *Pfhrp3* genes

Samples	No of samples	<i>Pfhrp2</i>	<i>Pfhrp3</i>	N=1724	95%CI	N=763	95%CI
<i>P. falciparum</i>	81	D	D	4.7%	3.8-5.8	10.6%	8.62-13
(N=122)	15	ND	ND	0.87%	0.53-1.43	1.97%	1.19-3.22
	11	D	ND	0.6%	0.36-1.14	1.4%	0.81-2.56
	15	ND	D	0.87%	0.53-1.43	1.9%	1.19-3.22
	92	D	NC	5.3%	4.37-6.5	12%	9.94-14.56
Mixed Infection (Pf/Pv)	1	D	D	0.06%	1e-04-0.33	0.1%	2e-04-0.74
(N=1)							

It is observed that in 81 samples appear deletion in both genes; 15 have no deletion in either of them, 11 have deletion only in *Pfhrp2*, 15 only in *Pfhrp3*. Regardless of what happens in *Pfhrp3* (whether there is or not deletion), there are 92 samples with deletion in *Pfhrp2*. In the mixed infection case, is detected deletion in both genes. N=1724 number of total samples, N=763 total of *P. falciparum* samples by PCR. D: deletion / ND: no deletion / NC: not considered

Figures

Figure 1

Map of the Continental Region of Equatorial Guinea. The Littoral Province where the sampling took place is highlighted. Source <https://www.cartedumonde.net>, modified. This map was used in Berzosa et al. J (2017) 16:28.

Figure 2

Sample Processing Flowchart. It is observed that 963 samples out 1724 were negatives by RDT and 761 positives. By NM-PCR was detected inside the negative group 128 positive samples (122 Pf, 1 Pm, 3 Po and 1 Pv/Pf), therefore, they were false negatives. The frequency of false negatives by RDT is 7.4%. In these 128 samples were studied the Pfhrp2 and Pfhrp3 genes. All these data are published in a previous article Berzosa P et al, Malar J. 2018 Sep 17;17(1):333.

Figure 3

Results of the Nested PCR for Pfdhfr, Pfdhps, Pfmdr1 and Pfcrf genes: amplification appears in all cases, therefore, it indicates that the DNA was well extracted and works correctly in PCR. These PCRs are used as a control, all samples amplified perfectly so when no amplification appears in pfhrp2 / 3 indicates for sure that there is deletion, it is not a problem with the DNA.

Figure 4

Results of the Nested PCR for Pfhrp2/3; the presence of the amplification fragment indicates the presence of the gene: figure A, lines 1 / 2 / 3 / 4 / 5 (+/-814bp for Pfhrp2) and figure B: lines 1 / 2 (+/-719bp for Pfhrp3). When the fragments do not appear indicate that deletion exists. ; fragments are sequenced to confirm that they correspond to the pfhrp2 / 3