IJID Regions 3 (2022) 300–307

Contents lists available at ScienceDirect

IJID Regions

journal homepage: www.elsevier.com/locate/ijregi

Plasmodium falciparum histidine-rich protein 2 and 3 gene deletion in the Mount Cameroon region

Esum Mathias Eyong[a,*], Sophie Jose Molua Etutu[a], Fru-Cho Jerome[a], Raymond Babila Nyasa[a], Tebit Emmanuel Kwenti[b], Marcel N. Moyeh[c]

[a] Department of Microbiology and Parasitology, Faculty of Science, University of Buea, P.O. Box 63, Buea, South West Region, Cameroon
[b] Department of Medical Laboratory Sciences, Faculty of Health Sciences, University of Buea, P.O. Box 63, Buea, South West Region, Cameroon
[c] Department of Biochemistry and Molecular Biology, Faculty of Science, University of Buea, P.O. Box 63, Buea, South West Region of Cameroon

ARTICLE INFO

Keywords:
Malaria
Plasmodium falciparum
Rapid Diagnostic Test
pfhrp2
pfhrp3
gene deletion

ABSTRACT

Objective: *Plasmodium falciparum* produces histidine-rich protein 2/3 (*Pfhrp2/3*) genes that accumulate to high levels in the bloodstream and serve as a diagnostic and prognostic marker for *falciparum* malaria. *Pfhrp2/3* gene deletions may lead to false-negative rapid diagnostic test (RDT) results. We aimed to determine the prevalence of *pfhrp2/3* gene deletions in *P. falciparum* isolates and the implications for RDT use in the Mount Cameroon region.
Methods: A cross-sectional hospital-based study with malaria diagnosis performed using microscopy, RDT and nested polymerase chain reaction (nPCR). In total, 324 *P. falciparum* microscopy positive individuals were enrolled and their samples confirmed positive for *P. falciparum* using 18SrRNA PCR. Samples that gave false-negative RDT results were analyzed to detect *pfhrp2/3* exon 2 deletions.
Results: Of 324 positive microscopic and nPCR samples, 16 gave RDT false-negative results. Among the 324 *P. falciparum* positive isolates, exon 2 deletions were observed in 2.2% (7 of 324); 3 were negative for *pfhrp2* gene, 2 for *pfhrp3*, and 2 for both *pfhrp2* and *pfhrp3* (double deletions).
Conclusion: *P. falciparum* isolates with *pfhrp2/3* gene deletion were present in the parasite populations and may contribute to the RDT false-negative results in the Mount Cameroon region.

1.1. Introduction

Malaria is an infectious disease with an estimated 229 million cases globally; it was responsible for 409,000 deaths in 2019 (World Health Organization, 2020). In the same year, *Plasmodium falciparum* malaria was responsible for nearly all malaria cases and fatalities in Sub-Saharan Africa and 94% of all malaria cases and deaths worldwide (World Health Organization, 2020). Malaria caused by the *falciparum* parasite continues to have a detrimental impact on human life and fragile economies.

Malaria remains prevalent in Cameroon, with an estimated 71% of the population living in high transmission areas (World Health Organization, 2011), and is the leading cause of morbidity and mortality amongst the most vulnerable groups, including children under the age of 5, pregnant women and the poor. Effective malaria therapy necessitates accurate laboratory diagnosis, which is still a key component of global malaria control efforts (Ali *et al.*, 2016).

Malaria rapid diagnostic tests (RDTs) based on histidine-rich protein-2 (*hrp2*) have improved malaria case management and surveillance, especially in Africa, where *P. falciparum* is most common. The emergence of gene deletion on *P. falciparum* histidine-rich proteins 2 (*pfhrp2*) and 3 (*pfhrp3*) has jeopardized the utility of these RDTs. Any *pfhrp2* or *pfhrp3* gene deletion may impact the performance of *PfHRP2*-based RDTs, leading to incorrect patient diagnosis and therapy. For reliable investigation, confirmation and reporting of *pfhrp2* and *pfhrp3* gene deletion, standard and recommended methods must be used. Agaba *et al.* (2019) and Kojom and Singh (2020) conducted systematic reviews and found unambiguous evidence of *pfhrp2* and *pfhrp3* gene deletion in Africa, where *P. falciparum* is the most common pathogen, and *HRP2*-based RDTs are the most utilized for malaria diagnosis. There is currently no information on the deletion of the *pfhrp2* and *pfhrp3* genes in Cameroon.

2.1. Materials and methods

2.1.1. Study design

We conducted a hospital-based cross-sectional study from August 2020 to July 2021 involving febrile patients seeking malaria diagnosis in the regional hospitals in Buea and Limbe in the Mount Cameroon Region.

Abbreviations: Pfhrp2, Plasmodium falciparum histidine-rich protein 2.; Pfhrp3, Plasmodium falciparum histidine-rich protein 3..

* Corresponding author.
E-mail addresses: eyong.mathias@ubuea.cm, mathias_mesum@yahoo.fr (E.M. Eyong).

https://doi.org/10.1016/j.ijregi.2022.05.006
Received 14 March 2022; Received in revised form 16 May 2022; Accepted 17 May 2022

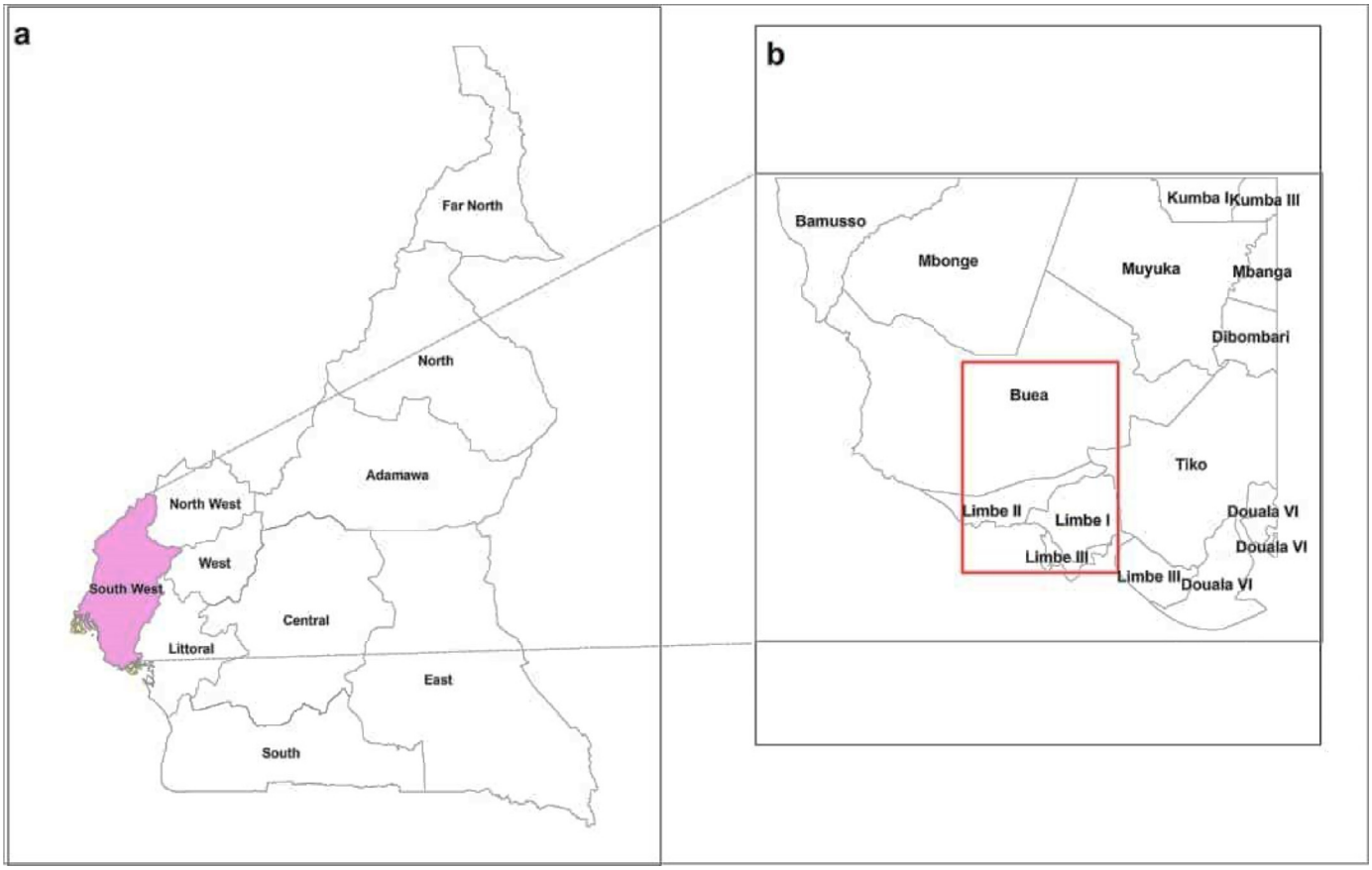

Fig. 1. Map showing study sites (not drawn to scale); a: Cameroon, b: South West Region. Source: Generated using ArcGIS version 10.2.2. (Colour)

2.1.2. Study area

Several studies in Africa have reported the presence of *pfhrp2/3* gene deletions. Our study was carried out in the Mount Cameroon region, situated in Fako Division (4°10′00″N, 9° 10′00″E) and covering an area of 2093 km^2 with an elevation ranging from 0 to 4095 m above sea level (Fig. 1). The region has an estimated population of 534,854 and the major towns include Buea, Idenau, Limbe, Mutengene, Muyuka and Tiko. The climate is generally hot and dry, except for Buea, which has a humid climate due to its location on the slopes of Mount Cameroon. Fako Division experiences two seasons, rainy and dry. The rainy season is usually between March and October, and the dry season is between November and February.

2.1.3. Study participants

Our study involved patients coming for consultation in the Out-Patient Department or Emergency Unit of the regional hospitals who consented to participate in the study. They were febrile patients of both sexes and all ages residing in the study area who presented with malaria symptoms but were not on any antimalarial medication.

2.1.4. Sampling technique

A simple random sampling technique was used to enrol participants with a sample size calculated using the formula described by Swinscow (2002):

$$n = \frac{(Z^2 xp(1-p)}{e^2} \text{ where}$$

n= sample size p= prevalence of deletion from previous study in Nigeria = 17% (Funwei *et al.*, 2019)

z = 95%CI = 1.96

e = error = 0.05

$$n = \frac{1.96^2 x \leq 0.17(1-0.17)}{0.05^2}$$

n = 217

Hence, we required approximately 300 participants to adjust for dropouts.

2.1.5. Data collection

A structured questionnaire was used to gather study participants' knowledge of malaria and preventive methods. Participants' axillary temperature was measured using an electronic thermometer; fever was defined as ≥37.6°C (Sumbele *et al.*, 2016).

2.1.6. Sample collection

Venous blood (2–5 mL) was collected from each participant and dispensed into ethylenediaminetetraacetic acid tubes using antiseptic techniques. The uncoagulated blood was used to perform the malaria diagnostic tests (microscopy, RDT and polymerase chain reaction [PCR]). Thick/thin blood smear and RDT for malaria were conducted for all participants. A few drops of blood were spotted on labelled filter paper (Whatmann_ No.3, Sigma-Aldrich, Germany) and allowed to air dry. The papers with dried blood spots were individually stored in plastic bags with a desiccant at room temperature for subsequent DNA extraction.

2.1.7. Sample analysis

2.1.7.1. Detection of malaria parasite by Giemsa microscopy. The blood films were prepared, air-dried and stained with freshly prepared 10%

Table 1
Primer sequences for amplification of 18SrRNA gene, pfhrp2 and pfhrp3 genes in malaria parasites

Species		Primer	Sequence (5'-3')	Expected fragment length in Base pairs (bp)
Plasmodium spp.		rPLU5	CCTGTTGTTGCCTTAAACTTC	900
		rPLU6	TTAAAATTGTTGCAGTTAAAACG	
P. falciparum		rFAL1	TTAAACTGGTTTGGGAAAACCAAATATATT	205
		rFAL2	ACACAATGAACTCAATCATGACTACCCGTC	
	Outer	Pfhrp2-F1	CAAAAGGACTTAATTTAAATAAGAG	
pfhrp2		Pfhrp2-R1	AATAAATTTAATGGCGTAGGCA	600 – 1000
	Nested	Pfhrp2-F2	ATTATTACACGAAACTCAAGCAC	
		Pfhrp2-R1	AATAAATTTAATGGCGTAGGCA	
	Outer	Pfhrp3-F1	AATGCAAAAGGACTTAATTC	
pfhrp3		Pfhrp3-R1	TGG TGTAAGTGATGCGTAGT	600 – 950
	Nested	Pfhrp3-F2	AAATAAGAGATTATTACACGAAAG	
		Pfhrp3-R1	TGG TGTAAGTGATGCGTAGT	

Giemsa solution for 25–30 min according to the standard World Health Organization (WHO) Methods Manual procedure (World Health Organization, 2015) and read separately by two expert microscopists. A third microscopist resolved any discrepancy between readings. Detection of malaria parasites and estimation of the parasite density by light microscopy was performed as described in the WHO Methods Manual (World Health Organization, 2015).

2.1.7.2. Diagnosis of malaria parasite by rapid diagnostic test. Two commercially available RDT kits, CareStart Malaria Pf/PAN (HRP2/pLDH) (ACCESSBIO, New Jersey, USA) Ag Combo RDT and SD BIOLINE Malaria Ag *P.f* (Standard Diagnostics Inc, Korea) RDT, were used to detect malaria parasites, according to the manufacturer's instructions.

The CareStart Malaria Pf/PAN (HRP2/pLDH) Ag Combo RDT is a chromatographic test for the rapid qualitative detection of malaria *hrp2* and pLDH (*plasmodium* lactate dehydrogenase) of *P. falciparum, P. vivax, P. ovale* and *P. malariae* in human whole blood. The RDT membrane strip is pre-coated with two monoclonal antibodies as two independent lines across the test strip. One line (test line 2) is pan-specific to pLDH of the *Plasmodium* species and the other line (test line 1) contains a monoclonal antibody specific to *P. falciparum* HRP2. Antibodies absorbed in gold particles are impregnated on the conjugate pad. The test had a sensitivity of 98% (95% CI 97.05%–100%) and specificity of 97.5% (95% CI 94.64%–99.36%).

The SD BIOLINE Malaria Ag *P.f* RDT is a fast chromatographic test for the qualitative detection of the *hrp2* antigen of malaria *P. falciparum* in human whole blood. On the test line region, a membrane strip is precoated with mouse monoclonal antibodies specific to HRP2 of *P. falciparum*. The malaria *P. falciparum* antigen in the specimen reacts with the mouse monoclonal antibodies specific to HRP2 of *P. falciparum* colloid gold conjugate. They chromatographically migrate through the membrane to the test region, where they form a visible line as an antibody-antigen-antibody gold particle complex with a high degree of sensitivity and specificity. The test had a sensitivity of 99.7% (95% CI 98.5%–100%) and specificity of 99.5% (95% CI 97.2%–99.9%).

2.1.7.3. Detection of malaria parasite by PCR.

2.1.7.3.1. Parasite DNA extraction. The DNA template for the nested PCR (nPCR) assay was extracted from dried blood on filter paper using the Chelex 100 method, as previously described (Musapa *et al.*, 2013). Then, 120 μL of the total genomic DNA was carefully transferred into sterile Eppendorf tubes and stored at -20°C for future use.

2.1.7.3.2 P. falciparum *identification by nested PCR.* Detection of malaria parasite DNA was based on nPCR amplification of the 18SrRNA gene in a reaction that used 2 μL of the extracted DNA, 7.5 μL of GoTaq polymerase and master mixes (Promega, USA), 0.5 μL each of upstream and downstream primers, and 4.5 μL of nuclease-free water in a total reaction volume of 15 μL. The first PCR included genus-specific primers, while the second nPCR run included *P. falciparum*-specific primers (species-specific primers), as previously described (Abdallah *et al.*, 2015), using the primary amplicons as the DNA template (Table 1). nPCR was repeated for all negative results. For discordant results, amplification was repeated a third time, with the final result being two consistent results. The thermocycling conditions were as shown in Table 2.

Table 2
Thermocycling conditions for amplification of Plasmodium falciparum

Steps	Cycling Conditions		
	Temperature		Time
	Primary PCR	Nested PCR	
Pre-denaturation	94°C		3 minutes
Denaturation	94°C		1 minute
Annealing	55°C	61°C	1 minute
Extension	68°C		1 minute
Number of Cycles	25	30	
Final extension	68°C		5 minutes
Hold	4°C		∞

2.1.7.3.3 nPCR amplifications of the pfhrp2 *and* pfhrp3 *genes.* Positive *P. falciparum* samples that were RDT-negative were used for further amplification of the exon 2 of *pfhrp2* and *pfhrp3* genes to detect the presence or absence of these two genes. The primary PCR amplification of *pfhrp2* and *pfhrp3* genes was performed using the primers and reaction conditions described in Tables 1 and 3. These amplifications were performed in a total volume of 15 µL consisting of 7.5 µL of One Taq 2X master mix with standard buffer, 0.5 µL each of outer and nested primers, 2 µL of DNA template and 4.5 µL of nuclease-free water. The nPCR was performed in the same manner with the amplicons from the primary reaction being used as the DNA template. nPCR was repeated for all negative results. In the instance of contradictory results, the amplification was repeated a third time, with the final result being two consistent results.

2.1.7.4. Confirmation of gene deletion. To verify the presence of parasites in the *pfhrp2*-negative samples, microscopy-positive samples with positive results for 18SrRNA but negative results for *pfhrp2/3* were considered *pfhrp2/3*-deleted after excluding low parasitaemia to avoid incorrect deletion calls.

2.1.7.5. Agarose gel electrophoresis. A 2% (w/v) agarose gel was used to confirm the presence of bands. The amplified PCR products (amplicons) were detected by running 10 μL of the PCR mixture on the agarose gel, stained with 0.5 μg/mL ethidium bromide solution. The samples were run using a Powerpack (Biorad, CA, USA) at 99 volts for 30 min alongside a 100 bp DNA ladder and a negative control (autoclaved distilled water), followed by separation. The bands were visualized under a UV transilluminator (Bio-Rad).

Table 3
Thermocycling conditions for amplification of Pfhrp2 and Pfhrp3 Genes

Species	Initial Denaturation	Denaturation	Annealing	Extension	Cycles	Final extension	Hold
Pfhrp2 Outer	94° for 5 minutes	94°C for 30 seconds	59°C for 1 minute	68°C for 1 minute	40	68°C for 3 minutes	4°C
Nested	94°C for 5 minutes	94°C for 30 seconds	59°C for 1 minute	68°C for 1 minute	40	68°C for 3 minutes	4°C
Pfhrp3 Outer	94°C for 5 minutes	94°C for 30 seconds	55°C for 1 minute	68°C for 1 minute	40	68°C for 3 minutes	4°C
Nested	94°C for 5 minutes	94°C for 30 seconds	55°C for 1 minute	68°C for 1 minute	40	68°C for 3 minutes	4°C

Table 4
Socio-demographic and Clinical characteristics of study participants

Characteristics	Variable	Number	Percentage
Gender	MaleFemaleTotalSex ratio	1232013240.61	38.062.0100
Age Group (Years)	< 2020-2930-3940-49≥50Total	11984422950324	36.725.913.09.015.4100
Mean ± SD ageMin – Max	27.45 ± 19.41-99		
Educational Level	Non-formal educationPrimarySecondaryTertiaryVocational trainingTotal	94115410119324	2.812.747.531.25.9100
Marital Status	SingleMarriedWidowedTotal	16414911324	50.646.03.4100
Temperature	37.5 – 38.338.4 – 39.4≥39.5Total	276399324	85.212.02.8100
Mean Temp.	37.9 ± 0.49		
Number of times infected	123>3Total	367758153324	11.123.817.947.2100
Parasitemia (T/μL)	< 10001000-49995000-99,999≥100,000Total	741371085324	22.842.333.31.5100
Mean density ± SDMin – Max	11 825.99 ± 33 033.24180 – 344 727		

Table 5
Microscopy, RDT, and nPCR results for detection of P. falciparum

Variable	Examined	Positive n (%)	Negative n (%)
Microscopy	324	324 (100)	0 (0.0)
RDT 1 (CareStart™ Malaria Pf/PAN)	324	308 (95.1)	16 (4.9)
RDT 2 (SD BIOLINE Malaria Ag *P.f*)	324	308 (95.1)	16 (4.9)
nPCR	324	324 (100)	0 (0.0)

Abbreviations: RDT, rapid diagnostic test; nPCR, nested polymerase chain reaction

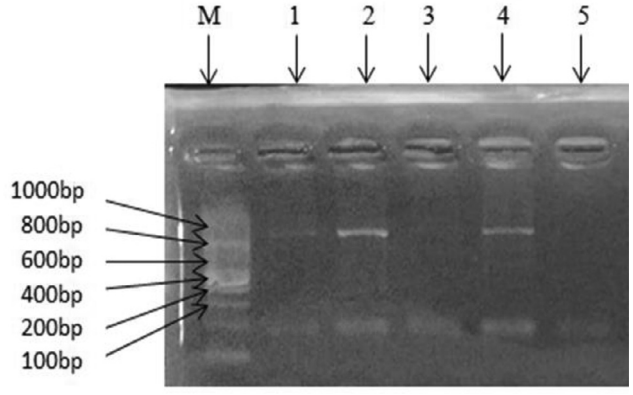

Fig. 2. Nested PCR of samples on gel representing *pfhrp2* genes M= molecular weight marker; 3 is the negative control; 5 is a negative sample; 1, 2, 4, are positive samples

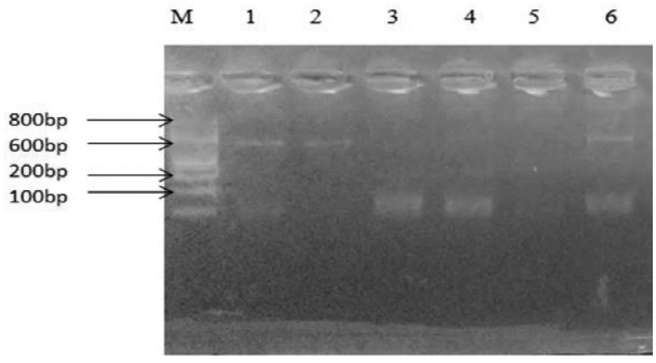

Fig. 3. Nested PCR of samples on gel representing pfhrp3 genes
M= molecular weight marker; 3 is the negative control; 5 is a negative sample; 1, 2, 4 and 6 are positive samples

2.1.7.6. Data analysis. Data collected were entered into Census and Survey Processing System (CsPro 7.6 software) and analyzed using IBM® SPSS® Statistics version 20 (IBM, USA) for Microsoft Windows. Pearson's chi-square test was performed. Data were double-checked to detect missing data or errors. The threshold for statistical significance was set at $P<0.05$.

3.1. Results

3.1.1. Sociodemographic and clinical characteristics of study participants

A total of 324 patients who were positive by microscopy for *P. falciparum* malaria were enrolled in the study: 201 (62.0%) were women and 123 (37.8%) men, 36% (119 of 324) were aged <20 years, 47.5% (154 of 324) had a secondary school level education, and 50.6% (164 of 324) were single. Table 4 provides the sociodemographic and clinical characteristics of the participants.

The mean body temperature of the participants was 37.9°C ± 0.49 (Table 4). All participants had a past malaria infection with a history of fever: 47.2% (153 of 324) had been infected >3 times between birth and when they were enrolled in this study and 58.3% (189 of 324) had received malaria treatments from health facilities.

3.1.3. Detection of P. falciparum malaria

Of the 324 microscopy positive samples, 308 (95.1%) were RDT-positive and 16

(4.9%) were RDT-negative. nPCR confirmed all samples were positive for *Plasmodium* infection. *P. falciparum* was the only species detected in the positive samples by both microscopy and PCR. Table 5 shows the results of malaria detection using microscopy, RDT and PCR.

Table 6
Diagnostic profile and parasite density of the microscopy positive/RDT negative isolates

SN	Sample ID	Microscopy	Results					
			RDT1	RDT2	nPCR	*hrp2*	*hrp3*	Parasites/μL
1	L002	+	-	-	+	+	+	3,200
2	L052	+	-	-	+	-	-	3,800
3	L069	+	-	-	+	+	+	240
4	L077	+	-	-	+	+	+	400
5	L094	+	-	-	+	+	+	880
6	L095	+	-	-	+	+	+	240
7	L102	+	-	-	+	-	-	3,200
8	L104	+	-	-	+	+	+	480
9	L128	+	-	-	+	-	+	780
10	B001	+	-	-	+	+	+	7000
11	B004	+	-	-	+	+	-	20,000
12	B005	+	-	-	+	+	+	16,000
13	B056	+	-	-	+	+	+	820
14	B114	+	-	-	+	-	+	180
15	B123	+	-	-	+	+	-	1592
16	B124	+	-	-	+	-	+	6766

Table 7
Prevalence of P. falciparum hrp2/hrp3 deletions in the false-negative isolates (n=16)

Variable	Number	Percentage (95% CI)
pfhrp2		
Positive	11	68.8 (43.8-87.5)
Negative	5	31.3 (12.5-56.3)
pfhrp3		
Positive	12	75.0 (50.0-93.8)
Negative	4	25.0 (6.3-50.0)

3.1.4. *Amplification of* pfhrp2 *and* pfhrp3 *genes*

Amplification of *pfhrp2* and *pfhrp3* genes was done on the 16 samples that were RDT negative but PCR positive. Of these samples, 2 were negative for both *pfhrp2* and *pfhrp3* genes (double deletions), 3 negative for *pfhrp2* genes only, and 2 negative for *pfhrp3* genes only. Figures 2 and 3 show the PCR gel banding patterns of the *pfhrp2* and *pfhrp3* genes.

Of the 16 samples, 11 (68.8%) were positive for *pfhrp2* genes and 12 (75.0%) were positive for *pfhrp3* genes. Nine had coexisting positive results for both *pfhrp2* and *pfhrp3* genes, 2 were positive only for *pfhrp2* genes and 3 were positive only for *pfhrp3* genes, as shown in Table 6.

Out of the 7 samples with *pfhrp2/3* gene deletions, PCR assay identified 3 (42.9%) as deleted for *pfhrp2* genes only, 2 (28.6%) as deleted for *pfhrp3* genes only, and 2 (28.6%) deleted for both *pfhrp2* and *pfhrp3* genes (fig. 4A).

3.1.5. *Prevalence of* pfhrp2 *and* pfhrp3 *gene deletions in the total isolates and the false-negative RDT samples*

Out of the 324 samples, the prevalence of *pfhrp2/3* gene deletion was 2.2% (7 of 324) (Fig. 4B). The prevalence of *pfhrp2* gene deletion was 1.5% (5 of 324) and 1.2% (4 of 324) for *pfhrp3* gene deletion, with 2 of these samples having deletions for both *pfhrp2* and *pfhrp3* genes (0.6% prevalence). Amplification of the *Plasmodium* 18SrRNA identified 16 samples to have false-negative RDT results. Of these 16 samples, 43.8% (7 of 16) had *pfhrp2/3* gene deletion (Fig. 4C), 31.3% (5 of 16) had *pfhrp2* gene deletion and 25.0% (4 of 16) had *pfhrp3* gene deletion (Table 7). A total of 2 isolates were negative for both *pfhrp2* and *pfhrp3* giving a prevalence of 12.5% for double deletions in the false-negative RDT isolates.

4.1. *Discussion*

In our study, 7 of the 324 participants had *pfhrp2/3* gene deletion giving a prevalence of 2.2%, similar to a study carried out in Mali which found a 2.0% prevalence. However, the prevalence in this study is far less than the 62.0% prevalence observed in Eritrea (Berhane et al., 2018). In the absence of the *pfhrp2* protein, most of the antibodies used in RDTs to detect *pfhrp2* also detect *pfhrp3* due to structural homology (Gendrot et al., 2019).

The prevalence of *pfhrp2* exon 2 deletions was 1.5% (5 of 324) in this study, which is comparable to previous studies in Senegal (2.4%), Mozambique (1.45%) and Ethiopia (4.8%) (Wurtz *et al.*, 2013; Gupta *et al.*, 2017; Girma *et al.*, 2018), and lower than findings reported in Ghana (36.2%, Amoah *et al.*, 2016), DR Congo (6.4%, Parr *et al.*, 2017), Kenya (9% Beshir *et al.*, 2017), Eritrea (9.7%, Menegon *et al.*, 2017), Rwanda (23%, Kozycki *et al.*, 2017), Sudan (60%, Hamid *et al.*, 2017), Eritrea (62%, Berhane *et al.*, 2018), Zambia (37.5%, Kobayashi *et al.*, 2019), Nigeria (17%, Funwei *et al.*, 2019) and Ethiopia (17.9%, Alemayehu *et al.*, 2021).

Similarly, the prevalence of *pfhrp3* exon 2 deletion (1.2%; 4 of 324) was comparable to studies in Kenya which found a prevalence of 1.1% (Beshir *et al.*, 2017) but lower than the prevalence of deletion reported in Honduras, 44.1% (Abdallah *et al.*, 2015), Eritrea 100% (Berhane *et al.*, 2018) and Ethiopia 9.2% (Alemayehu *et al.*, 2021). Variations in transmission intensity, geographical location, sample size, and laboratory methods used to analyze *pfhrp2/3* genes deletions could explain these discrepancies.

In the present study, 47.8% (7 of 16) of *Pf*HRP2 RDT negative samples lacked exon 2 of *pfhrp2/3*, the main amino acid coding region of the *pfhrp*2 and *pfhrp*3 gene. Of these PCR-negative pfhrp*2/3* exon 2 samples, all were microscopy positive. The *pfhrp2/3* gene deletions observed in this study would undoubtedly impact the diagnostic performance of *Pf*HRP2 RDTs. The WHO's threshold level for *pfhrp2/3* deletions causing false-negative RDT results is much lower at 5% (World Health Organization, 2018). Our study also revealed that 68.75% (11 of 16) of false-negative *PfHRP2* RDT results were *pfhrp*2 exon 2 positive. The cause of these false-negative *Pf*HRP2 RDT results could, as reported in previous studies, be due to the absence of *Pf*HRP2 antigen as a result of host immune response in high transmission areas (Ho *et al.*, 2014), variation in the amino acid sequence expressing reduced levels of the target antigens (Cheng *et al.*, 2014), or the genetic variation of the *pfhrp2* gene leading to a modified protein *Pf*HRP2 with a new epitope that is not recognized by RDT antibodies (Gendrot *et al.*, 2018).

The proportion of isolates with *pfhrp2* gene deletions among the false-negative cases using *Pf*HRP2-based RDT was 31.3% (5 of 16). A similar result (23%) was reported in Ghana (Amoah *et al.*, 2016). Conversely, only 2.4%, 10.6% and 9.9% of false-negative *Pf*HRP2-RDT results involved parasites with *pfhrp2* gene deletions were reported in Senegal (Wurtz *et al.*, 2013), Nigeria (Funwei *et al.*, 2019) and DR Congo (Munyeku *et al.*, 2021), respectively. Similarly, the prevalence of isolates

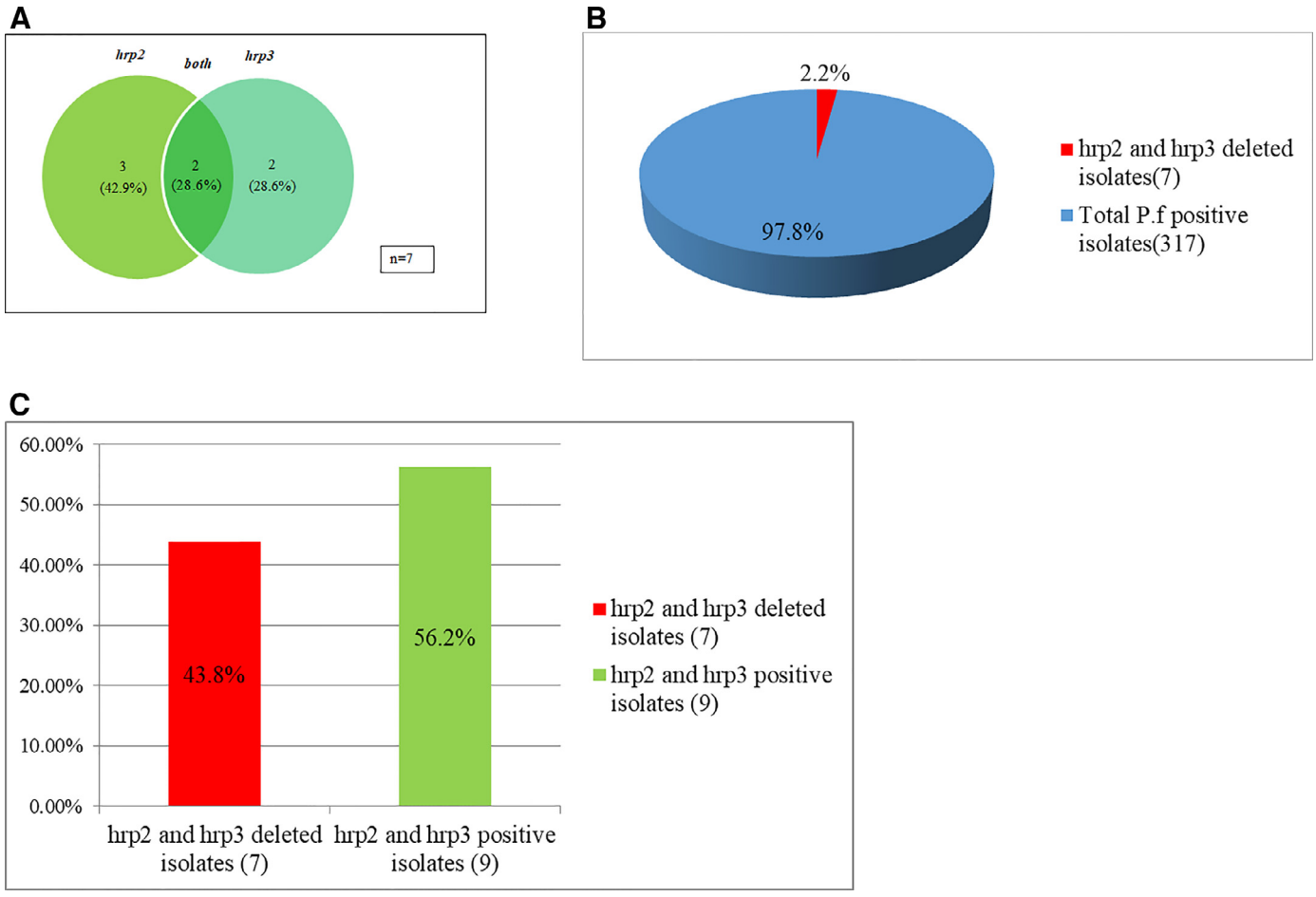

Fig. 4. Representations of Frequency and Prevalence of deletions; A: Venn diagram demonstrating frequencies of pfhrp2/pfhrp3 gene deletions (in number and percentage), B: Prevalence of pfhrp2 and pfhrp3 gene deletions in P. falciparum Isolates, C: Representation of prevalence of P. falciparum hrp2 and hrp3 gene deletions in RDT false-negative samples. (Colour)

with *pfhrp3* gene deletions among the false-negative cases using *Pf*HRP2-based RDT was 25.0% (4 of 16), differing from the 85.8% prevalence (6 of 7) recorded in Senegal (Wutz *et al.*, 2013).

Genetic alterations in parasites, such as *pfhrp*2/3 deletion, are unlikely to be the only source of RDT false negatives. Some of the RDT false-negatives samples were found to be positive using nPCR and there were no identifiable deletions in the *pfhrp2/3* genes. These findings could be attributed to issues with the RDT employed or operator error in completing the tests and/or interpreting the results, which could lead to false negatives, as documented in prior research (Berzosa *et al.*, 2020).

Partially deleted *pfhrp2* and *pfhrp3* genes, prozone effects due to abundant antigen, sequence variability of *pfhrp*2 and *pfhrp*3 genes, and circulating antibodies to HRP2 have all been observed to interfere with HRP2 RDT detection (Gamboa *et al.*, 2010; Lee *et al.*, 2006; Ho *et al.*, 2014). Although the primers employed in this investigation only amplified exon 2 of the *pfhrp2* gene and exon 2 of the *pfhrp3* gene, the *pfhrp2* and *pfhrp3* genes are known to contain chromosomal breaking sites outside of exon 2 (Cheng *et al.*, 2014). In individuals with hyperparasitaemia, HRP2-based RDTs have been reported to show prozone-like effects. Although the mechanism of prozone-like effects for antigen detection assays is unknown, one likely explanation is that the amount of antigen detected exceeds the dye-labeled antibodies' binding capability. Unlabeled target antigen reaches the test strip in this case, saturating the binding capacity of the test strip's capture antibodies. As a result, dye-labeled antibodies that capture antigen may fail to bind to the test strip and generate a visible band (Luchavez *et al.*, 2011). However, the prozone impact has only been linked to false-negative HRP2 test lines in samples with ≥288,000 parasites/μL (Gillet *et al.*, 2011). Therefore, based on our parasite density data, it is unlikely to have been a significant cause of false-negative RDTs in this study.

This study mentions the role of an intact *pfhrp3* gene in PfHRP2 RDT-positive results. Although only three of the *pfhrp3* exon 2 PCR positive samples lacked *pfhrp*2 exon 2, these three samples were confirmed positive by PCR and microscopy in the absence of the *pfhrp2* gene, suggesting that *pfhrp3* exon 2 may have contributed. This finding aligns with previous studies (Amoah *et al.*, 2016; Beshir *et al.*, 2017; Alemayehu *et al.*, 2021) which showed that structural similarities between epitopes of PfHRP2 and PfHRP3 antigens allow PfHRP2 monoclonal antibodies to cross-react with PfHRP3 (Lee *et al.*, 2012). Indeed, the true prevalence of *pfhrp2* gene deletion in our study may have been underestimated by PfHRP2 RDT positive test results with intact *pfhrp3* exon 2.

Our findings demonstrate that *pfhrp2*-negative parasites are more common in our study site than *pfhrp3*-negative parasites, which has also been seen in Suriname (Akinyi Okoth *et al.*, 2015). In Colombia, Peru and Honduras (Gamboa *et al.*, 2010; Akinyi *et al.*, 2013; Abdallah *et al.*, 2015), *pfhrp3*-negative parasites outnumber *pfhrp2*-negative parasites.

5.1. Conclusion

Our study revealed that *P. falciparum* isolates with *pfhrp2* and *pfhrp3* gene deletions are present in the parasite populations in the Mount Cameroon region. False-negative RDT results may lead to misdiagno-

sis of patients with malaria infections where the diagnosis is based on the use of *Pf*HRP2-based RDTs only.

It is essential to have good diagnostic tools on the front line to control malaria. Therefore, there is a need for more extensive studies involving samples collected from different geographical settings across Cameroon to estimate the true prevalence of pfhrp2/3 deleted parasites and their impact on malaria diagnosis in Cameroon.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors' contributions

EME: Methodology, supervision, formal analysis, writing – original draft of the manuscript. **SJME:** Conceptualization, methodology, data collection, review and editing. **FCJ:** Co-supervision, review and editing. **RBN:** Co-supervision, review and editing. **TEK:** Data collection, editing. **MNM:** Resources, review. All authors discussed the results and contributed to the final manuscript. All authors read and approved the final manuscript.

Acknowledgments

We acknowledge the financial support of the Faculty of Science, University of Buea Research Grant 2019/2020. We thank the Directors and laboratory staff of the Buea Regional Hospital Annex and Limbe Regional Hospital for facilitating data collection at study sites. We thank all the study participants.

Availability of data and materials

All data and materials supporting the results of the manuscript are available from eyong.mathias@ubuea.cm / mathias_mesum@yahoo.fr upon request.

Consent for publication

All authors have seen and approved the final version of the manuscript for publication in the International Journal of Infectious Diseases.

Ethics approval and consent to participate

Ethical clearance for this study was obtained from the Institutional Review Board of the Faculty of Health Sciences, the University of Buea, Cameroon (Ref: 2021/1255-11/UB/SG/IRB/FHS). Authorization was obtained from the Ministry of Public Health, Regional Delegation for the Southwest Region (Ref: R11/MINSANTE/SWR/RDPH/PS/240/986). Further administrative authorization was obtained from the Medical Directors of the Regional Hospital Buea Annex and the Regional Hospital Limbe. Written informed consent was obtained from all participants ≥18 years of age, and parents or legal guardians of children <18 years gave written informed consent on behalf of their children before their inclusion in the study.

Funding

The work was supported by the Faculty of Science, University of Buea Research Grant 2019/2020.

References

Abdallah JF, Okoth S, Fontecha GA, Torres R, Banegas EI, Matute M, Bucheli S, Goldman IF, de Oliveira A, Barnwell JW, Udhayakumar V. Prevalence of pfhrp2 and pfhrp3 gene deletions in Puerto Lempira, Honduras. Malar J 2015;14:19. doi:10.1186/s12936-014-0537-7.

Agaba BB, Yeka A, Nsobya S, Arinaitwe E, Nankabirwa J, Opigo J, Mbaka P, Lim CS, Kalyango JN, Karamagi C, Kamya MR. Systematic review of the status of pfhrp2 and pfhrp3 gene deletion, approaches and methods used for its estimation and reporting in Plasmodium falciparum populations in Africa: review of published studies 2010–2019. Malar J 2019;18(1):355. doi:10.1186/s12936-019-2987-4.

Akinyi Okoth S, Abdallah JF, Ceron N, Adhin MR, Chandrabose J, Krishnalall K, Huber CS, Goldman IF, Macedo de Oliveira A, Barnwell JW, Udhayakumar V. Variation in Plasmodium falciparum Histidine-Rich Protein 2 (Pfhrp2) and Plasmodium falciparum Histidine-Rich Protein 3 (Pfhrp3) Gene Deletions in Guyana and Suriname. PLoS One 2015;10(5). doi:10.1371/journal.pone.0126805.

Akinyi S, Hayden T, Gamboa D, Torres K, Bendezu J, Abdallah JF, Griffing SM, Quezada WM, Arrospide N, De Oliveira AM, Lucas C, Magill AJ, Bacon DJ, Barnwell JW, Udhayakumar V. Multiple genetic origins of histidine-rich protein 2 gene deletion in Plasmodium falciparum parasites from. Peru. Sci Rep. 2013;3:2797. doi:10.1038/srep02797.

Alemayehu GS, Blackburn K, Lopez K, Cambel Dieng C, Lo E, Janies D, Golassa L. Detection of high prevalence of Plasmodium falciparum histidine-rich protein 2/3 gene deletions in Assosa zone, Ethiopia: implication for malaria diagnosis. Malar J 2021;20(1):109 https://doi.org/https://doi.org/. doi:10.1186/s12936-021-03629-x.

Ali IM, Bigoga JD, Forsah DA, Cho-Ngwa F, Tchinda V, Moor VA, Fogako J, Nyongalema P, Nkoa T, Same-Ekobo A, Mbede J, Fondjo E, Mbacham WF, Leke RGF. Field evaluation of the 22 rapid diagnostic tests for community management of malaria with artemisinin combination therapy in Cameroon. Malar J 2016;15:31. doi:10.1186/s12936-016-1085-0.

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. doi:10.1186/s12936-016-1159-z.

Berhane A, Anderson K, Mihreteab S, Gresty K, Rogier E, Mohamed S, Hagos F, Embaye G, Chinorumba A, Zehaie A, Dowd S, Waters NC, Gatton ML, Udhayakumar V, Cheng Q, Cunningham J. Major Threat to Malaria Control Programs by Plasmodium falciparum Lacking Histidine-Rich Protein 2. Eritrea. Emerg Infect Dis. 2018;24(3):462–70 https://doi.org/https://doi.org/. doi:10.3201/eid2403.171723.

Berzosa P, González V, Taravillo L, Mayor A, Romay-Barja M, Garcia L, Ncogo P, Riloha M, Benito A. First evidence of the deletion in the pfhrp2 and pfhrp3 genes in Plasmodium falciparum from Equatorial Guinea. Malar J 2020;19(1):99. doi:10.1186/s12936-020-03178-9.

Beshir KB, Sepúlveda N, Bharmal J, Robinson A, Mwanguzi J, Busula AO, de Boer JG, Sutherland C, Cunningham J, Hopkins H. Plasmodium falciparum parasites with histidine-rich protein 2 (pfhrp2) and pfhrp3 gene deletions in two endemic regions of Kenya. Sci Rep 2017;7(1):14718 https://doi.or/10.1038/s41598-017-15031-2.

Cheng Q, Gatton ML, Barnwell J, Chiodini P, McCarthy J, Bell D, Cunningham J. Plasmodium falciparum parasites lacking histidine-rich protein 2 and 3: a review and recommendations for accurate reporting. Malar J 2014;13:283. doi:10.1186/1475-2875-13-283.

Funwei R, Nderu D, Nguetse CN, Thomas BN, Falade CO, Velavan TP, Ojurongbe O. Molecular surveillance of pfhrp2 and pfhrp3 genes deletion in Plasmodium falciparum isolates and the implications for rapid diagnostic tests in Nigeria. Acta Trop 2019;196:121–5. doi:10.1016/j.actatropica.2019.05.016.

Gamboa D, Ho M-F, Bendezu J, Torres K, Chiodini PL, Barnwell JW, Incardona S, Perkins M, Bell D, McCarthy J, Cheng Q. 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(1):e8091. doi:10.1371/journal.pone.0008091.

Gendrot M, Fawaz R, Dormoi J, Madamet M, Pradines B. Genetic diversity and deletion of Plasmodium falciparum histidine-rich protein 2 and 3: a threat to diagnosis of P. falciparum malaria. Clin Microbiol Infect 2019;25(5):580–5. doi:10.1016/j.cmi.2018.09.009.

Gillet P, Scheirlinck A, Stokx J, De Weggheleire A, Chauque HS, Canhanga OD, Tadeu BT, Mosse CD, Tiago A, Mabunda S, Bruggeman C, Bottieau E, Jacobs J. Prozone in malaria rapid diagnostics tests: how many cases are missed? Malar J 2011;10:166. doi:10.1186/1475-2875-10-166.

Gupta H, Matambisso G, Galatas B, Cisteró P, Nhamussua L, Simone W, Cunningham J, Rabinovich NR, Alonso P, Saute F, Aide P, Mayor A. Molecular surveillance of pfhrp2 and pfhrp3 deletions in Plasmodium falciparum isolates from Mozambique. Malar J 2017;16(1):416. doi:10.1186/s12936-017-2061-z.

Hamid MA, Awad-Elgeid M, Nasr A. Gene Variation And Suspected plasmodium Falciparum histidine-Rich Protein 2 Gene Deletion And Its Impact On Sensitivity Of Malaria Rapid Diagnostic Tests In Sudan. Bmj Global Health 2017;2(Suppl 2) A21.2A21.. doi:10.13140/RG.2.2.30281.36968.

Ho M-F, Baker J, Lee N, Luchavez J, Ariey F, Nhem S, Oyibo W, Bell D, González I, Chiodini P, Gatton ML, Cheng Q, McCarthy JS. Circulating antibodies against Plasmodium falciparum histidine-rich proteins 2 interfere with antigen detection by rapid diagnostic tests. Malar J 2014;13(1):480. doi:10.1186/1475-2875-13-480.

Kobayashi T, Sikalima J, Parr JB, Chaponda M, Stevenson JC, Thuma PE, Mulenga M, Meshnick SR, Moss WJ. & For The Southern And Central Africa International Centers Of Excellence For Malaria Research. The search for plasmodium falciparum histidine-rich protein 2/3 Deletions in Zambia and Implications for Plasmodium falciparum histidine-rich protein 2-Based Rapid Diagnostic Tests. Am J Trop Med Hyg 2019;100(4):842–5. doi:10.4269/ajtmh.18-0859.

Kojom LP, Singh V. Prevalence of Plasmodium falciparum field isolates with deletions in histidine-rich protein 2 and 3 genes in context with sub-Saharan

Africa and India: a systematic review and meta-analysis. Malar J 2020;19(1):46. doi:10.1186/s12936-019-3090-6.

Kozycki CT, Umulisa N, Rulisa S, Mwikarago EI, Musabyimana JP, Habimana JPet al. False-negative malaria rapid diagnostic tests in Rwanda: impact of Plasmodium falciparum isolates lacking hrp2 and declining malaria transmission. Malar J 2017;16(1):123. doi:10.1186/s12936-017-1768-1.

Lee N, Gatton ML, Pelecanos A, Bubb M, Gonzalez I, Bell D, Cheng Q, McCarthy JS. Identification of Optimal Epitopes for Plasmodium falciparum Rapid Diagnostic Tests That Target Histidine-Rich Proteins 2 and 3. J Clin Microbiol 2012;50(4):1397–405. doi:10.1128/JCM.06533-11.

Luchavez J, Baker J, Alcantara S, Belizario V, Cheng Q, McCarthy JS, Bell D. Laboratory demonstration of a prozone-like effect in HRP2-detecting malaria rapid diagnostic tests: implications for clinical management. Malar J 2011;10:286 2011https://doi.org/. doi:10.1186/1475-2875-10-286.

Menegon M, L'Episcopia M, Nurahmed AM, Talha AA, Nour BYM, Severini C. Identification of Plasmodium falciparum isolates lacking histidine-rich protein 2 and 3 in Eritrea. Infect Genet Evol 2017;55:131–4. doi:10.1016/j.meegid.2017.09.004.

Munyeku YB, Musaka AA, Ernest M, Smith C, Mansiangi PM, Culleton R. Prevalence of Plasmodium falciparum isolates lacking the histidine rich protein 2 gene among symptomatic malaria patients in Kwilu Province of the Democratic Republic of Congo. Infect Dis Poverty 2021;10(1):77. doi:10.1186/s40249-021-00860-1.

Musapa M, Kumwenda T, Mkulama M, Chishimba S, Norris DE, Thuma PE, Mharakurwa SA. Simple Chelex Protocol for DNA Extraction from Anopheles spp. J Vis Exp 2013;71:3281. doi:10.3791/3281.

Parr JB, Verity R, Doctor SM, Janko M, Carey-Ewend K, Turman BJ, Keeler C, Slater HC, Whitesell AN, Mwandagalirwa K, Ghani AC, Likwela JL, Tshefu AK, Emch M, Juliano JJ, Meshnick SR. Pfhrp2-Deleted Plasmodium falciparum Parasites in the Democratic Republic of the Congo: A National Cross-sectional Survey. J Infect Dis 2017;216(1):36–44. doi:10.1093/infdis/jiw538.

Sumbele IUN, Sama SO, Kimbi HK, Taiwe GSMalaria. Moderate to Severe Anaemia, and Malarial Anaemia in Children at Presentation to Hospital in the Mount Cameroon Area. Anemia 2016 2016. doi:10.1155/2016/5725634.

Swinscow TDV. Campbell MJ. Statistics at square. 10th edition. London: BMJ books; 2002.

World Health Organization & UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in *Tropical Diseases*, 2015. Microscopy for the detection, identification and quantification of malaria parasites and stained thick and thin blood films in research settings (version 1.0): procedure: methods manual, Geneva. https://apss.who.int/iris/handle/10665/163782/ (accessed 03 March 2020)

World Health Organization, 2011. World Malaria Report 2011. Geneva. http://www.who.int/malaria/world_malaria_report_2011/en/index.html/ (accessed 24 November 2020).

World Health Organization, 2018. Protocol for estimating the prevalence of pfhrp2/pfhrp3 gene deletions among symptomatic falciparum patients with false-negative RDT results. Geneva. http://www.who.int/malaria/publications/atoz/hrp2-deletionprotocol/en/ (accessed 27 April 2020). Licence: CC BYNC-SA 3.0 IGO.

World Health Organization, 2020. World Malaria Report; WHO: Geneva, Switzerland. https://www.who.int/publications/i/item/9789240015791/ (accessed 8 December 2020).

Wurtz N, Fall B, Bui K, Pascual A, Fall M, Camara C, Diatta B, Fall KB, Mbaye PS, Diémé Y, Bercion R, Wade B, Briolant S, Pradines B. Pfhrp2 and pfhrp3 polymorphisms in Plasmodium falciparum isolate from Dakar, Senegal: impact on rapid malaria diagnostic tests. Malar J 2013;12:34. doi:10.1186/1475-2875-12-34.