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Full Length Research Article

IMPACT OF LOW PARASITAEMIA ON THE SENSITIVITY OF *P. FALCIPARUM PFHRP2*-BASED RAPID DIAGNOSTIC TEST IN BENUE STATE NIGERIA

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ABSTRACT
Most reported sensitivities of *P. falciparum* specific malaria rapid diagnostic tests (RDTs) do not account for the integrity of the *PfHRP2* gene that produces the *HRP2* antigen in *P. falciparum* infections. However, low sensitivity of RDTs could be due to the deletion of this gene. This study aimed to determine the sensitivity and specificity of *P. falciparum* specific HRP2-based RDT, investigate levels of parasitaemia and deletion of *HRP2* gene in the parasites. A total of 272 children aged 0-14 years were diagnosed for malaria using CareStart Pf HRP2 malaria RDT, microscopy and nested species-specific malaria PCR. The level of parasitaemia, malaria species composition, and *pfHRP2* gene integrity of false negative malaria RDTs were also determined. The sensitivity of the RDT was 43.3%, 95%CI (37.7-50.3) versus microscopy, and 51.0%, 95%CI (40.5-61.3) versus PCR used as reference tests. Out of 47 false negative malaria RDTs, 100% were positive by *P. falciparum* species specific PCR, and had intact *PfHRP2* gene. The geometric mean parasitaemia of the false negative RDT parasites was 178 parasites/µl of blood versus 939 parasites/µl of blood in true positive cases. Thus, the low sensitivity of the Pf HRP2 malaria RDT was not due to deletion of the *pfHRP2* gene or the presence of non HPR2 producing malaria species. It was likely due to the low parasitaemia observed among the *P. falciparum* parasites.

Keywords: Malaria, Diagnosis, Rapid diagnostic test, Microscopy, PCR.

INTRODUCTION

The adoption of malaria rapid diagnostic tests (RTDs) for malaria diagnosis has facilitated early detection and timely treatment of malaria infection in many cases (Cunningham, *et al*., 2019). However, in some cases, positive diagnoses by malaria RDTs that were treated as malaria were not a true consequence of malaria infection (Hosch *et al*., 2022). The utilization of RDTs for malaria diagnosis and treatment has without doubt contributed immensely in the reduction of malaria morbidity in recent times, while saving lives that would have been exposed to increased risk of severe malaria infection and its adverse effects due to delayed treatment of unconfirmed cases of severe *P. falciparum* infections (Mousa, *et al.,* 2020; Borgstein *et al*., 2022). Most health care facilities in malaria endemic areas have reduced the use of microscopy for malaria diagnosis, relying mainly on malaria RDTs for diagnosis and treatment of malaria (Fitri *et al.,* 2022). Although malaria RDTs have continued to be a useful tool in this regard, a number of factors lower the sensitivities of these RDTs, leading to false negative test results (Iwuafor *et al*., 2018; Watson *et al*., 2019; Ajakaye and Ibukunoluwa, 2020). Low sensitivity of malaria RDTs could be due to low levels of parasitaemia below the limit detectable by malaria RDTs, deletion of the gene producing the antigen that is detected by the malaria RDT (Feleke *et al*., 2021; Kavanaugh *et al.,* 2021; Martiáñez-Vendrell *et al.,* 2022; Ikegbunam *et al*., 2023) or the adverse effect of unacceptable storage condition of the malaria RDTs in use (Albertini *et al*., 2012; Martiáñez-Vendrell *et al.,* 2022). Although some of these factors may impinge upon the sensitivities of malaria RDTs, they may not always affect the diagnostic ability of the malaria RDTs in equal measures in a local malaria transmission environment (Albertini *et al*., 2012). If the major factors that play a part in lowering the sensitivities of malaria RDTs are identified in local malaria transmission settings, and progressively scaled up to a country level, it will provide useful data in national malaria control programs. Specifically, it will provide useful data for formulating policies to guide the rational utilization of malaria RDTs in the diagnosis of malaria, for the treatment of the disease at local health facilities within a country.

In this study we determined the sensitivity and specificity of *P. falciparum* species specific malaria RDT that uses HRP2 antigen by substituting both microscopy diagnosis and malaria species specific nested PCR diagnosis, as reference tests. In addition, we sought to understand whether false negative cases of *P. falciparum* malaria observed with *PfHRP2* malaria RDT were due to the presence of other malaria species in our sample apart from *P. falciparum*. Moreover, the levels of parasitaemia, or cases of deletion of *HRP2* gene in the malaria parasites were also investigated in this study.

MATERIALS AND METHODS

Study Area

Samples for the study were collected at the Benue State Epidemiology unit in Makurdi, General Hospitals in Katsina-Ala and Buruku in Benue State.

Ethics Approval and Informed Consent

Ethical approval for this study was obtained from the Ethical Review Committee of the Benue State Ministry of Health and Human Services, with Reference number MOH/STA/204/VOL.1/79. Informed consent was sought and obtained from all the guardians or parents of the children who signed an informed consent form, after the objectives of the study and the protocol for sample collection were explained to them.

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Subject Recruitment and Blood Sample Collection for Malaria Diagnosis

Subjects enrolled in this study were children ≤ 14 years who sought health care at General Hospitals in the sampled areas, whose parents or guardian gave written informed consent. During blood sample collection, information on age, sex, body temperature and history of fever of each enrolled child were obtained. Finger prick blood samples were collected from June 2017 to December 2020, in the wet and dry seasons in each sampling location. Blood samples were obtained from children 0-14 years old with or without symptom of malaria from a total pool of 1089 children. A subtotal of 25% of the total number of samples collected (*n*=272) were randomly selected and analysed with three different malaria diagnostic tests namely, RDT, microscopy, and malaria species specific nested PCR.

Malaria Diagnosis with *P. falciparum* Histidine Rich Protein 2 Rapid Diagnostic Test

Finger prick blood from each child was collected and tested for malaria using the malaria rapid diagnostic test CareStart malaria *Pf (HRP2)* Ag RDT, manufactured by AccessBio, New Jersey USA. A positive or negative reaction of the malaria antigen to the RDT was obtained following the manufacturer's recommendation.

Microscopy Diagnosis of Malaria

A thick blood film was also prepared from the finger pricked blood and stained with 10% Giemsa stain for a period of 10 minutes. It was examined under the microscope using oil immersion at 100 X objective to determine the presence or absence of malaria parasites. Where malaria parasites were present, quantification of parasite load was performed based on standard procedures (WHO, 2016).

Blood Sample Collection for PCR Diagnosis

A few drops of the finger pricked blood were also collected on 3 mm Whatman filter paper (Bereczky *et al*., 2005), air dried and preserved with a desiccant as dry blood spot (DBS). Then malaria parasite DNA was subsequently extracted for PCR analysis from the DBS (Apinjoh *et al*., 2024).

Extraction of Malaria Parasite DNA

The extraction of malaria parasite was performed on n = 272 samples of randomly selected DBS from both symptomatic and asymptomatic children (Bereczky *et al*., 2005). The Zymo DNA mini extraction kit was used to extract the malaria parasite DNA. Briefly, a 3 mm^2 punch of the DBS was made with a paper punch. The punched DBS was further divided into smaller bits and transferred into a 1.5 ml DNase and RNase free microcentrifuge tube. The tissue lysis and DNA extraction protocol of the Zymo DNA mini kit, with silica spin columns was then applied to lyse, extract, purify, and elute the parasite DNA as detailed by the manufacturer's protocol. The eluted DNA was stored at -20 °C until PCR analysis.

Diagnosis of Malaria Parasites Using Species Specific Nested PCR

The PCR analysis for the detection of malaria species followed a slight modification of the malaria species specific nested PCR protocol previously described (Snounou and Singh, 2002), for the identification and differentiation of malaria species. The modification included the introduction 0.6% of bovine serum albumin (BSA) in the PCR master mix to enhance reactivity. For the primary reaction to determine the presence of malaria species, a total of 25.0 µL of PCR reaction mixture consisted of 18.65 µL of double distilled water, 1X standard PCR buffer containing 1.5 mM of $MgCl_2$, 200 µM of deoxy nucleoside triphosphates, 0.2 µM of each forward and reverse primers, 0.6 % volume of BSA, 1.0 unit of Taq DNA polymerase, and 2.0 µL of DNA template. All the PCR reagents used were purchased from New England Biolabs, USA, except the primers which were synthesized by Inqaba Biotec South Africa.

The PCR cycling conditions were 95 °C/3 minutes, followed by 35 cycles of 94 °C/30 seconds, 58.3 °C/30 seconds, 72°C/1 minute, 45 seconds, and the final extension at 72 °C/5 minutes. For the secondary reaction to detect specific species of malaria, a 1:10 dilution of the primary amplicon was used as DNA template. All the volumes of the PCR reaction mixture remained the same as in the primary reaction. For the cycling conditions, the number of cycles was reduced to 30, the annealing temperature was kept at 61.3 °C/30 seconds, the extension time was reduced to 50 seconds at 72 °C, all other cycling condition remained the same as in the primary reaction. The amplification was performed on GTS-9612 thermal cycler (Table 1).

Table 1: Primers Sequences used in the species identification of malaria parasites by species specific nested PCR and amplicon sizes

Primer Name	Sequence 5'-3'	Malaria Species and Amplicon Size (Base Pairs)
rPLU1	TCAAAGATTAAGCCATGCAAGTGA	Malaria species
rPLU5	CCTGTTGTTGCCTTAAACTTC	1.6 – 1.7 kb
rFAL1	TTAAACTGGTTTGGGAAAACCAAATATATT	*P. falciparum*
rFAL2	ACACAATGAACTCAATCATGACTACCCGTC	206 bp
rOVA1	ATCTCTTTTGCTATTTTTTAGTATTGGAGA	*P. ovale*
		226 bp
rPLU2	ATCTAAGAATTTCACCTCTGACATCTG	
rMAL1	ATAACATAGTTGTACGTTAAGAATAACCGC	*P. malariae*
		145 bp
rMAL2	AAAATTCCCATGCATAAAAAATTATACAAA	
rVIV1	CGCTTCTAGCTTAATCCACATAACTGATAC	*P. vivax*
		121 bp
rVIV2	ACTTCCAAGCCGAAGCAAAGAAAGTCCTTA	

Source: Snounou and Singh (2002)

Amplification of *PfHRP2* Gene

To investigate whether the deletion of *P. falciparum* histidine rich protein 2 gene was responsible for false negative samples which were positive by PCR, but negative by the CareStart HRP2 based malaria RDT; the amplification of the *PfHRP2* gene was carried out. The gene produces the HRP2 antigen which is used to diagnose *P. falciparum* malaria by the RDT. A set of published primers *For*: 5'- ATTCCGCATTTAATAATAACTTGTGTAGC-3' and *Rev*: 5'-ATGGCGTAGGCAATGTGTGG -3' were used for the amplification of the *PfHRP2* (exon 2) of the gene (Koita *et al*., 2012; Parr *et al*. 2018). The PCR conditions were similar to those already described (Parr *et al*., 2019). A deleted HRP2 gene from a sample that was false negative by malaria RDT used in the study, but was identified as true positive *P. falciparum* parasite by species specific nested PCR should yield no amplification of the HRP2 gene fragment. However, if the HRP2 gene of that *P. falciparum* parasite was not deleted, the gene fragment should be amplified by the primers.

Electrophoresis of PCR amplicons

The amplified PCR fragments were electrophoresed on 2 % agarose gel in TBE buffer at 95 millivolts for 45 minutes. The gels were stained with ethidium bromide, viewed on a UV-transilluminator (Analytik Jena), and photographed with a 13-megapixel camera for visual inspection and scoring of positive and negative bands.

Data Analysis

Data obtained from the Carestart malaria RDT test, and microscopy, and PCR diagnosis were analysed statistically on line, using the clinical research calculator 1, at www.vasarstat.com to determine malaria prevalence, sensitivity - which is the conditional probability that a specific test will be positive if malaria parasites were present. Also, the specificity which is the probability that a test will be negative if malaria parasites were absent, the positive and negative predictive values of RDT vs microscopy, RDT vs PCR and microscopy vs PCR were equally estimated. Both microscopy and PCR were substituted as a reference tests. In each case, a diagnostic parameter value and its 95 % confidence interval were estimated. For comparing the level of agreement between two diagnostic tests, *kappa* was estimated (Landis and Koch, 1977). The geometric mean parasitaemia of true positive and false negative malaria cases were also computed for comparison. In terms of parasite density, the geometric mean was used to estimate parasite load per microlitre of blood of CareStart malaria RDT negative, samples which were positive by microscopy and PCR, as well as RDT positive samples that were both microscopy and PCR positive.

RESULTS

The diagnosis of *P. falciparum* malaria by CareStart malaria RDT gave 1/272 (0.4%) invalid case. The invalid case was therefore removed, leaving 271/272 (99.6%) of cases for meaningful comparison. The prevalence of malaria among the children using each of the three different diagnostic methods was determined along with the 95% confidence intervals. The performance of the malaria RDT in detecting *P. falciparum* malaria infection was 20.7%; 38.2% by microscopy and 35.3% by PCR. Thus, the prevalence of *P. falciparum* in the sample using CareStart malaria RDT was lower than both microscopy and PCR (Table 2).

Table 2: Prevalence of malaria in children by RDT and Microscopy and by PCR (n=272)

Test	Positive/total observed	% Prevalence (95% CI)
RDT	56/271	20.7 (16.1 - 26.1)
Microscopy	104/272	38.2 (32.4 – 44.3)
PCR	96/272	35.3 (29.7 – 41.3)

The test agreement of pair wise combination of the three diagnostic methods based on *Kappa* values were: *kappa* = 0.40 or fair agreement of malaria RDT vs microscopy, 0.52 or moderate agreement of malaria RDT vs PCR, and 0.54 or moderate agreement of microscopy vs PCR (Table 3).

Table 3: Comparative Diagnosis of malaria by Carestart RDT, microscopy, and PCR

RDT	Microscopy (reference test)		Total *	*kappa*
	Positive	Negative		
Positive	45	11	56	
Negative	59	156	215	0.40
Total	104	167	271	
RDT	**PCR (reference test)**		**Total***	***Kappa***
	Positive (%)	**Negative (%)**		
Positive (%)	49	7	56	
Negative (%)	47	168	215	0.52
Total	96	175	271	
Microscopy	**PCR (reference test)**		**Total**	***Kappa***
	Positive	**Negative**		
Positive	71	33	104	
Negative	25	143	168	0.54
Total	96	176	272	

*: 1 invalid RDT was removed

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The sensitivity of RDT vs PCR as gold standard was 51.0%. This was lower than microscopy versus PCR as gold standard at 74.0%. Specificity of RDT versus PCR = 96.0% was higher than microscopy vs PCR = 81.3%. The positive predictive value of RDT vs PCR = 87.5% was also higher than microscopy vs PCR = 68.2. While for negative predictive value, microscopy vs PCR = 85.1% was higher compared to RDT vs PCR = 78.1%. Microscopy vs PCR had higher false positive predictive values = 31.7% compared to RDT vs PCR = 12.5%. The false negative predictive values of RDT vs PCR = 21.9% was higher than microscopy versus PCR = 14.9%, (Table 4).

Table 4: Comparative Diagnostic parameters of Carestart malaria RDT with microscopy and PCR (the reference/gold standard test has asterisk - *)

Test parameter	Comparative tests	Estimated value %	95% Confidence Interval	
			Lower limit %	Upper limit %
Sensitivity	RDT/microscopy	43.3	33.7	53.3
	RDT/PCR*	51.0	40.7	61.3
	Microscopy/PCR*	74.0	63.8	82.1
Specificity	RDT/microscopy	93.4	88.2	96.5
	RDT/PCR*	96.0	91.6	98.2
	Microscopy/PCR*	81.3	74.5	86.6
PPV	RDT/microscopy	80.4	67.2	89.3
	RDT/PCR*	87.5	75.3	94.4
	Microscopy/PCR*	68.2	58.3	76.9
NPV	RDT/microscopy	72.6	66.0	78.3
	RDT/PCR*	78.1	71.9	83.4
	Microscopy/PCR*	85.1	78.6	90.0
FPPV	RDT/microscopy	19.6	10.7	32.8
	RDT/PCR*	12.5	5.6	25.7
	Microscopy/PCR*	31.7	23.1	41.7
FNPV	RDT/microscopy	27.4	21.7	34.0
	RDT/PCR*	21.9	16.6	28.1
	Microscopy/PCR*	14.9	10.0	21.4

Key:
PPV = positive predictive value; **NPV** = negative predictive value, **FPPV** = false positive predictive value, **FNPV** = false negative predictive value

The geometric mean parasite density/µL of blood of false negative RDT samples that were both microscopy and PCR positive was 178 parasite/µL. this was nearly 5 times lower than the corresponding value of 939 parasites/µL for blood samples that were RDT, microscopy and PCR positive (Fig. 1). The analysis of PCR amplicon by gel electrophoresis showed the predominant presence of *P. falciparum* malaria (Plates 1 and 2, Fig.2). Only a single case of *P. ovale* was identified, the remaining cases were *P. falciparum*. Among the false negative RDT cases which were positive both by microscopy and PCR that were examined for *PfHRP2* gene deletion, all the gene fragments with variable sizes amplified (Plate 3). This demonstrated that there was no single case of *PfHRP2* deletion observed among the samples examined.

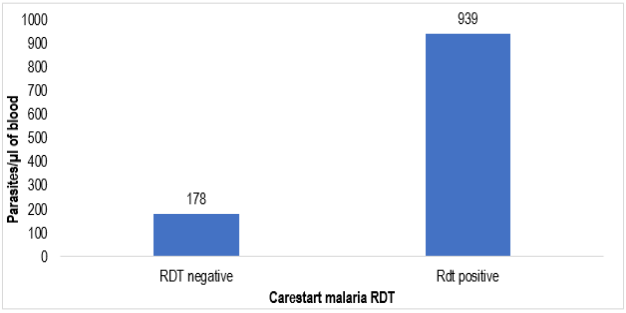

Fig 1: Geometric mean parasite density/µL of blood of false negative RDT, microscopy and PCR positive (n = 47) and RDT, microscopy and PCR positive samples (n = 49)

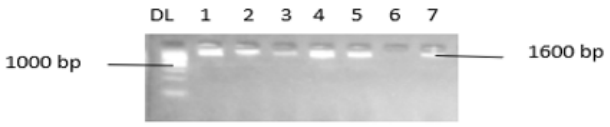

Plate 1: PCR amplified 1600 bp fragments of malaria species

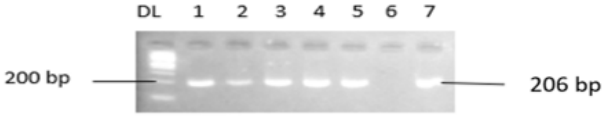

Plate 2: PCR Amplified 205 bp fragments of *P. falciparum* malaria

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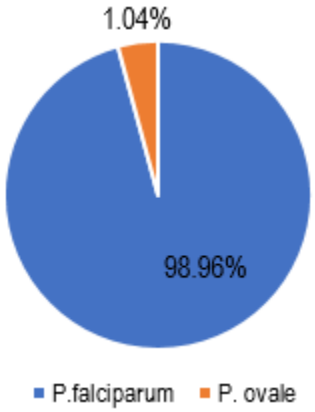

Fig 2. Malaria species composition identified in the study (n = 96).

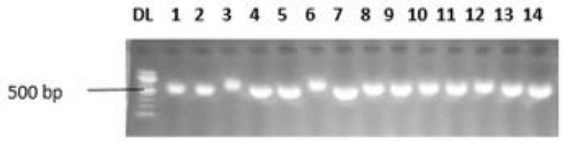

Plate 3: PCR amplified variable fragment sizes of *PfHRP2* gene from false negative CareStart malaria RDT samples

DISCUSSION

Reduced sensitivity of *P. falciparum* specific HRP2 malaria RDTs have been reported in some *P. falciparum* endemic localities in recent years (Danwang *et al*., 2012; Thomson *et al.,* 2019; Slater, *et al*., 2022; Kayode *et al*., 2024). The observation of 51.1% sensitivity of similar RDT type in this study vs PCR, used as a gold standard is therefore in sync with results obtained elsewhere in Nigeria and indeed many areas in Africa where *P. falciparum* is endemic. In a different case, high sensitivity of *PfHPRP2* and *PfLDH* malaria RDTs were reported in Ethiopia (Alemayehu *et al*., 2020), suggesting differential sensitivities to malaria diagnosis with RDTs which might be due to prevailing factors, indigenous to a malaria endemic locality. In this study, the reduced sensitivity of the HRP2 RDT in use led to the inability of the kit to accurately diagnose over 20% cases of malaria that microscopy diagnosis was able to identify. Consequently, there was a need to explain in particular, the false negative malaria cases that were classified by the HRP2 malaria RTD.

There are many variables that can interfere with the accurate diagnosis of HRP2 malaria RDTs (Martiáñez-Vendrell *et al*., 2022). In our study, the species composition of malaria parasites, the integrity of the HRP2 gene, and the level of parasitaemia were considered. Among these, species specific analysis of malaria parasites, by PCR determined that save for a single case of *P. ovale* that was present in the PCR positive samples (*n* = 96), 98.96% of the malaria parasites were positively diagnosed as *P. falciparum*. The single case of *P. ovale* was among the 48.9% (47) cases of false negative RDT samples, and was correctly not detected by the RDT. This is true since *P. ovale* does not encode the HRP2 gene (Baker *et al*., 2010), that produces the HRP2 antigen, which *P. falciparum* specific malaria RDTs detect. The remaining 46 cases of RDT false negatives were detected by PCR as *P. falciparum* parasites. However, the *P. falciparu*m specific malaria RDT deployed in this study failed to accurately detect them as *P. falciparum* malaria cases, leading to lower than expected sensitivity of the CareStart malaria RDT. Thus, the low sensitivity observed in this study was not due to significant presence of other species of malaria that lack the production of HRP2 antigen, which the CareStart RDT is not design to detect.

Among the five known malaria parasite species that can infect humans, only *P. falciparum* encodes a gene for the production of HRP2 (Baker *et al*., 2010). A fact that its gene product is used for the selective diagnosis of *P. falciparum* while, unable to detect the other malaria species. There are indications from several malaria endemic regions around the world that, the deletion of HRP2 gene compromises the ability of HRP2 specific malaria RDTs in detecting the presence of *P. falciparum* (Gendrot *et al.,* 2019). Although empirical evidence of HRP2 deletion among parasites originated from South America (Gamboa *et al*., 2010), supporting evidence have also emerged from malaria endemic regions such as Rwanda and the Democratic Republic of Congo (Gendrot *et al.,* 2019; Kayode *et al.,* 2024), Tanzania (Thomson *et al*., 2019), Uganda (Thomson *et al*., 2019; Bosco *et al*., 2020), and Nigeria (Ikegbunam, *et al*., 2024). In *PfHRP2* gene deleted *P. falciparum* malaria isolates, malaria diagnosis with a HRP2 RDT kit would be ineffective against real *P. falciparum* parasites. As a consequence, it would yield high cases of false negative *P. falciparum* malaria parasite as observed in our study. To confirm if this was the likely situation in our observation, we used published primers to amplified the exon 2 of HRP2 gene (Koita et al., 2012; Parr *et al*., 2018) of all the 46 cases of false negative parasites that were identified as *P. falciparum* positive cases by malaria species specific PCR (Snounou and Singh 2002). The resultant amplification of variable size fragments of the *PfHRP2* gene in each of the false negative RDT parasites did not support any evidence of gene deletion. This is different from reports by other researcher in some malaria settings (Gendrot *et al*., 2019; Alemayehu *et al*., 2020; Feleke *et al*., 2021). We concluded that *PfHRP2* gene deletion did not contribute to the low sensitivity of HRP2 malaria RDT observed in our study.

The insignificant production of HRP2 antigen by low levels of parasitaemia in the blood may contribute to false negative cases of *P. falciparum* malaria. Even in the absence of PfHRP2 gene deletion, when diagnosis is performed with the PfHRP2 malaria RDT kit on actual *P. falciparum* parasites. In Uganda for instance, false negative cases of similar nature were not due to deletions of *HPR2* and *HRP3* genes (Nsobya *et al*., 2020). Also, in Ghana (Thomson *et al*., 2019; and in Tanzania (Bakari *et al*., 2020) there were no cases of gene deletion. In the present study, the geometric mean parasitaemia of the false negative malaria cases was a mere 178 parasites/microlitre of blood. This was 5 times lower than that of malaria positive cases identified by the same RDT, microscopy and PCR. This observation suggests that low parasitaemia was the primary cause of the inability of the HRP2 malaria RDT used in this study to accurately detect *P. falciparum* malaria positive cases.
Very low parasitaemia may hinder the production of sufficient HRP2 antigen levels in the blood, below a certain threshold for RDT detection, leading to failure of the RDT to diagnose positive *P. falciparum* malaria cases. There is supporting evidence in some Angolan patients where it was found that a substantial proportion of persons with blood HRP2 antigen concentrations not detected by the malaria RDT had evidence of active infection by quantitative reverse transcription-PCR (qRT-PCR), with low parasite density

levels (Plucinski *et al*., 2019). In Burundi, The LoD, defined as the lowest parasite density that could be detected with 95% probability, was 178 parasites/µL of blood with 95%CI:(94-339) parasite, for the CareStart HRP2 Pf malaria RDT (Niyukuri *et al*., 2022). Thus, the low geometric mean parasite density of 178 parasites/µL of blood; observed among the false negative cases of CareStart HRP2 malaria RDT in our study may explain why these positive cases of malaria were not accurately detected by the HRP2 RDT. Despite the absence of *PfHRP2* gene among the parasites.

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