

Original Article

Comparative Evaluation of Three Histidine-Rich Protein-2 Based Rapid Diagnostic Tests, Microscopy and PCR for Guiding Malaria Treatment in Ibadan, Southwest Nigeria

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ABSTRACT

Background: Malaria rapid diagnostic tests (mRDTs) are the preferred option for programmatic deployment. **Aims:** There are numerous mRDTs on the Nigerian market and there is a need to guide practitioners on the relative performance of the commonly used brands of mRDT in Nigeria. **Subjects and Methods:** The performance of three commonly used Histidine-Rich-Protein-2-based mRDTs (SD-Bioline™, Carestart™ and Paracheck-Pf™) against microscopy of Giemsa stained blood and polymerase chain reaction (PCR) was evaluated among 190 febrile under-5 children in Ibadan, Nigeria. We calculated the sensitivity, specificity, predictive values, accuracy, and agreements. **Results:** There were 53.2% males. The prevalence of malaria parasite by microscopy was 46.8% and 57.9% by PCR. Malaria parasite detection by SD-Bioline™ was 60.5%, Carestart™: 60.0% and Paracheck-Pf™ 60.0%. Using microscopy as the gold standard, the sensitivities of SD-Bioline™, Carestart™ and Paracheck-Pf™ mRDT were 97.8%, 96.7% and 97.8% respectively while the specificities were 73.0%, 72.0% and 74.0% respectively. Using PCR as the gold standard, the sensitivity for both SD-Bioline™ and Paracheck-Pf™ was 85.5% and for CareStart was 84.6% while the specificity of SD-Bioline™, Carestart™, and Paracheck-Pf™ was 73.8%, 72.4%, and 75.0% respectively. The test accuracy was 81.0% for both SD-Bioline™ and Paracheck-Pf™ and 80.0% for Carestart™. The kappa coefficient of agreement between PCR and each of SD-Bioline™, Carestart, ParaCheck™ and microscopy was 0.597, 0.578, 0.609 and 0.739 respectively. **Conclusion:** The performance of the three mRDTs is a proof that any of the three is suitable for use in the diagnosis of malaria in the southwest of Nigeria.

KEYWORDS: HRP-2
Malaria-RDTs,
microscopy, Nigeria,
PCR

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INTRODUCTION

The 2018 World Malaria Report^[1] shows that nearly half of all malaria cases occurred in five nations: Nigeria (25%), the Democratic Republic of the Congo (11%), Mozambique (5%), India (4%), and Uganda (4%). Like many other endemic countries of the world, children under 5 years of age are the most vulnerable group affected by malaria in Nigeria.^[1] Although efforts to address this vulnerability through the distribution of insecticide-treated bed nets, scale-up of malaria rapid diagnostic tests and artemisinin-based combination therapy in high-risk groups are on-going, the burden remains unacceptably high.^[2]

The current recommendation by the WHO is that all cases of suspected malaria fever should be confirmed with a parasite-based test such as microscopy or mRDT before treatment.^[3] Although a presumptive diagnosis of malaria is inaccurate, it is still widely practiced by health workers despite the introduction of parasite-based diagnosis into the Nigeria national malaria treatment guideline.^[4] The consequences of this practice include indiscriminate use of antimalarial drugs and unnecessary drug pressure. These could lead to the emergence of resistance to the few available antimalarial drugs, increased cost of care and failure or delay in making the correct diagnosis and appropriate treatments.^[5,6]

The WHO-recommended procedure for confirming the diagnosis of all suspected cases of malaria are microscopy and malaria rapid diagnostic tests (mRDTs).^[3] The main malaria antigens used in mRDTs are histidine-rich protein-2 (HRP2), parasite-specific lactate dehydrogenase (pLDH), and aldolase.^[7] Histidine-rich protein-2 is a *P. falciparum*-specific antigen, while pLDH and aldolase are common to all *Plasmodia* species.^[7,8] Some of the strengths and weaknesses of microscopy and RDTs described in the literature are focused on sensitivity, specificity, predictive values, cost-effectiveness, the need for microscopists, and the question of technicians' experience.^[9,10]

However, the conventional microscopic diagnosis is considered as the gold standard for health facility management of malaria cases but it has many challenges. The staining and interpretation processes are labour-intensive, time-consuming and require electricity, skilled-manpower and considerable experience for the accurate identification especially at low parasitaemia or mixed malaria species.^[11] Although an expert microscopist can detect parasitaemia as low as 5 parasites/ μ l and an average microscopist can detect 50-100 parasites/ μ l, the lack of access to this expertise

in resource-limited settings limits the use of microscopy in routine clinical practice at the primary care level.^[10,11]

Malaria RDTs are a good alternative to microscopy in settings where microscopy is unavailable or not feasible. Malaria RDTs have a major advantage over microscopy in that they require little or no equipment or expertise and provide results promptly in under 30 minutes which makes it more user-friendly and less laborious.^[12] Several mRDTs are currently in use to detect malaria parasites, but there are variations between products, although the test principles are similar. According to the WHO, mRDT procurement should be based on product performance, especially in the context of the population in which it will be used.^[13] Some studies in Nigeria have assessed and reported the performance of these mRDTs as varying from poor through moderate to very good performance.^[13-20] Notably, the differences in the findings of these studies can be attributed to the difference in patients population, choice of the gold standard, and malaria transmission status.^[12,21]

Test performance accuracy is very critical when selecting a malaria screening or diagnostic tool, as the best mRDTs are those that produce valid and consistently reproducible results when compared to the gold standard.^[22] Since the identification of parasite at very low parasite density is dependent on the expertise of the microscopist, molecular techniques with high sensitivity and accuracy such as nested Polymerase Chain Reaction (PCR), are gaining more attention. In this study, we evaluated the performance of the commonly used three brands of HRP-2 based mRDTs against microscopy and PCR in case management of malaria in rural and urban areas of Ibadan in southwest Nigeria.

MATERIALS AND METHODS

Study design

The study was cross-sectional in design, nested into a larger cohort study^[23] which evaluated the consequences of restricting artemisinin-based combination therapy (ACT) treatment to mRDT positive in children less than 5 years, as stipulated by the National Malaria Treatment Guidelines. The data reported in this paper are those 190 out of 511 children from the larger study.^[23]

Study site and patients

This study was conducted in a rural primary healthcare center (Idi-Ayunre), in Oluyole Local Government area and a secondary healthcare facility (St. Mary Catholic hospital) in Ibadan South-east local government area in the southwest of Nigeria. Malaria RDTs were performed and read according to the manufacturer's

instructions at the study site and treatment decisions were based on mRDT result.^[23] Consecutive 190 children, aged 3 to 59 months, with complaints of fever (axillary temperature $\geq 37.5^{\circ}\text{C}$) at enrolment or a history of fever in the 48 hours preceding presentation were enrolled in the study. Enrollees with danger signs were excluded from the study. Children who tested positive to mRDT obtained standard doses of artesunate/amodiaquine (ASAQTM; Sanofi Aventis, Bridgewater, NJ, USA. ASAQ) according to body weight) ASAQTM as described earlier.^[23]

Laboratory procedures

Capillary blood from a finger prick was used to perform RDT after cleaning with 70% alcohol, prepare thick blood smears on microscope slides, and collect blood spots on filter paper (WhatmanTM 3 MM) for PCR analysis. The three *PfHRP-2* based mRDT brands evaluated during this study are SD-BiolineTM (Standard Diagnostic Inc., Korea), CarestartTM (Access Bio, Inc. USA) and ParacheckTM (Orchid Biomedical Systems, India). The test preparation and interpretation were done according to the manufacturer's instructions. Reading and interpretation of test results were carried out by two trained research assistants and interpreted by consensus within the specified 15–20 min test window. When the test and control lines were visible in their respective test windows, the test was considered positive, negative when only the control band was visible and invalid when the control band was not visible. Also considered positive were faint test lines.

Dried blood smears were stained with 10% Giemsa following standard procedure. Thick films used for parasite identification and quantification were screened by two expert microscopists who were blind to the mRDT result later in the laboratory at the University College Hospital (UCH), Ibadan, Nigeria. A thick blood smear was considered negative only after 100 consecutive high-power fields were screened and were free of the malaria parasite. Parasite density was calculated by counting the number of asexual parasites against about 200 leukocytes, assuming a leukocyte count of 8,000/ μL .^[24,25]

Finger-prick blood was blotted on the WhatmanTM 3 MM filter paper, dried in a dust-free area away from the sun and subsequently placed in a zip-lock bag with silica gel to prevent DNA contamination and degradation. At the end of the study, dried blood spots on the filter were processed in the Molecular Biology laboratory of the Department of Medical Microbiology and Parasitology, Ladoke Akintola University of Technology, (LAUTECH) Osogbo, South Western Nigeria for DNA isolation and PCR amplification.

The DNA was extracted from filter paper using QIAamp DNA Mini kit blood and tissue (QIAGEN Hilden Germany) according to the manufacturer's instructions. The extracted DNA was stored at -20°C until use. DNA samples were amplified by species-specific primer pairs [Additional File 1] to amplify the *P. falciparum*, *P. vivax*, *P. malariae* and *P. ovale* genes of the small subunit ribosomal ribonucleic acid (ssrRNA).^[26] Both primary and nested amplifications were performed in a 20 μL reaction volume containing 2 μL 10 $\times$ PCR buffer, 0.8 μL MgCl_2 , 0.2 μL dNTPs, 0.5 μL primers, 0.2 μL Taq DNA-polymerase, 10.8 μL nuclease-free PCR grade water and 5 μL DNA template on a thermal cycler (Prime Thermal-cycler Germany). The primary *Plasmodium* genus-specific amplification reaction was followed by nested *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* species-specific PCR amplification. The first amplification thermo-cycling conditions were: Initial denaturation at 94°C for 5 minutes, followed by 25 cycles, respectively, 1 min at 94°C for denaturation, 2 min at 58°C for annealing temperature, and 2 min at 72°C extension. The thermal conditions for the nested PCR were: Initial denaturation at 94°C for 5 min, followed by 30 cycles of 1 min for denaturation at 94°C , 2 min for annealing temperature at 58°C , and 2 min for annealing at 72°C . The final extension was 5 min at 72°C for both primary and nested reactions. Negative and positive controls have been included in each PCR run. After staining with SybrTM Green, amplicons were separated on a 1.5% agarose gel electrophoresis run alongside a 100 bp DNA ladder (New England Biolabs UK). The presence or absence of various species of *Plasmodium* was confirmed with a species-specific representative amplicon base pair size.

Data management and statistical analysis

Using the microscopy and PCR as the gold standard, the sensitivity, specificity, positive and negative predictive values of the three HRP2-based mRDTs were calculated with EpiCalc software (version 2000).^[27] The agreement between diagnostic methods was assessed by calculating the *kappa* statistics (*k*) and the corresponding standard error of the mean.^[28] A *k*-value of 0.40 to 0.60 was considered moderate agreement while 0.61 to >0.80 were considered substantial to almost perfect agreement. Data were analysed using IBM SPSS for Windows 20.0 for analysis. Statistical significance was set at $P = 0.05$.

Ethical consideration

Ethical approval for the study was obtained from the University of Ibadan/University College Hospital Institutional Review Committee, Oyo State Ministry of Health, Institutional Review Committee (IRC), and The London School of Hygiene and Tropical Medicine IRC.

Written informed consent or witnessed oral informed consent was obtained from each child's parent or guardian before any study-related procedure.

RESULTS

Characteristics of enrollees

The study participants comprised of 115 and 75 febrile children from rural and urban communities, respectively. There were 101 (53.3%) males, 89 (46.8%) females, and the mean age of enrollees was 24 ± 13 months for males and 24 ± 17 months for females. All enrollees had a history of fever within 48 hours of presentation, but only 109 (57.4%) had an axillary temperature of $\geq 37.5^\circ\text{C}$ at enrolment. The average weight for males was 11.31 ± 3.34 kg and 10.68 ± 3.69 kg for females [Table 1].

Malaria parasite detection by microscopy, malaria RDTs, and PCR

Less than half, 46.8% (89/190) of the enrollees had patent parasitaemia detected by microscopy. The geometric mean parasite density was $11,229/\mu\text{L}$ with a range of 28–611,600/ μL while the median was 20,800/ μL . Six (6.7%) of the enrollees with patent parasitaemia had parasite density $<200/\mu\text{L}$, 4 (4.5%) had parasite density between 200 and 500/ μL while 88.8% (79/89) recorded parasite density $>500/\mu\text{L}$. The prevalence of malaria parasite detection by SD-Bioline™ was 60.5% (115/190) while that of CareStart™ and Paracheck™ was 60% (114/190). Polymerase chain reaction detected malaria parasite in 57.9% (110/190) of the enrollees. On speciation, *Plasmodium falciparum* was the most prevalent specie (83.6%; 92/110) followed by *Plasmodium malariae* (20.9%; 23/110) and *Plasmodium ovale* (6.4%; 7/110). Thirteen of 23 (56.5%) of the *P. malariae* infections occurred as mono-infection while the remaining 10 (43.5%) occurred as co-infection with *P. falciparum*. Similarly, 57.1% (4/7) of *P. ovale* infections occurred as mono-infection while 42.9% (3/7) occurred as co-infection with *P. falciparum*.

Performance of the three malaria Rapid diagnostic tests

The performance of the three MRDTs using microscopy as gold-standard is shown in Tables 2-4. CareStart™ recorded one case of an invalid test result. There were only two cases of false-negative MRDT results with SD-Bioline™ and Paracheck™ while CareStart™ had three false-negative results. All three MRDT brands recorded false-positive results at parasite density of 514/ μL and 5,538/ μL while CareStart™ recorded the third false negative at 28/ μL . On the other hand, there were 28, false-positive results with SD-Bioline™ and Paracheck™ while there were 27 false positives with CareStart™.

Table 1: Demographic and clinical Characteristics of febrile children evaluated for malaria by three HRP2-based malaria rapid diagnostic tests

Characteristics	Number (n=190)	Percentage
Study Site		
Rural (Idi-ayunre)	115	60.5
Urban (St Mary's Hospital, Eleta)	75	39.5
Overall total	190	100
Sex		
Male	101	53.2
Female	89	46.8
Temperature in $^\circ\text{C}$		
Mean $\pm$ sd	39.9 $\pm$ 1.2	-
Range	36-40.9	-
$\geq 37.5^\circ\text{C}$	109	57.4
Weight in kilograms		
Mean $\pm$ SD	11.02 $\pm$ 3.51	-
Range	4.8-27	-
Malaria parasite detection		
By microscopy Day 0	89	46.8
By mRDT at Day 0		
SD-Bioline™ - +ve	115	60.5
Care Start™ - +ve	114	60.0
Paracheck™ - +ve	114	60.0
By PCR on day 0	110	57.9
Malaria Parasite Speciation (n=110)		
<i>P. falciparum</i>	92	83.6
<i>P. malariae</i>	23	20.9
<i>P. ovale</i>	7	6.4
<i>P. vivax</i>	Nil	Nil

*Median=20,800/ μL (range=28-611,600/ μL)

Table 2: Details of SD-Bioline™, CareStart and ParaCheck™ MRDT Performance against Microscopy

Parameter	SD-Bioline™	CareStart	ParaCheck™
True Positive	87	86	87
False Positive	28	28	27
True Negative	73	72	74
False Negative	2	3	2
Invalid	NIL	1	NIL
Total	190	190	190

Using microscopy as the gold standard, sensitivity, specificity, positive predictive value (PPV), negative predictive value and accuracy were 97.8%, 72.7%, 76.5% and 97.3%, respectively for SD-Bioline™, 96.7%, 72.7%, 76.3% and 96.0%, respectively for CareStart™, and 97.8%, 73.7%, 77.2%, and 97.3%, respectively for ParaCheck™. Table 3 also shows that the accuracy of SD-Bioline™, CareStart™ and ParaCheck™ were 85.3%, 84.2%, and 85.4%, respectively compared with expert microscopy. Also, the estimates of the agreement and between microscopy and mRDT results were as shown in

Table 3: Performance of three Malaria Rapid Diagnostic Tests against Microscopy as Gold Standard

Parameter	Malaria Rapid Diagnostic Test Brand		
	SD-Bioline™	CareStart™	ParaCheck™
Sensitivity	97.8%	96.7%	97.8%
Specificity	72.7%	72.7%	73.7%
Positive predictive value	76.5%	76.3%	77.2%
Negative predictive values	97.3%	96.0%	97.3%
Accuracy	85.3%	84.2%	85.4%
κ- statistics	0.696	0.686	0.707
*p	<0.001	<0.001	<0.001
*OR (95% CI)	13.50 (3.13, 82.09)	9.0 (2.61, 37.19)	13.0 (3.01, 79.9)

κ-Kappa, *McNemar Chi square test was used

Table 4: Performance of three Malaria Rapid Diagnostic Tests and Microscopy against PCR as Reference Standard

Parameter evaluated	Malaria Rapid Diagnostic Test Brand			Microscopy
	SD-Bioline™	Care Start™	Paracheck™	
Sensitivity	85.5%	84.6%	85.5%	79.0%
Specificity	73.4%	73.4%	75.0%	97.5%
Positive predictive value	81.7%	81.6%	82.5%	97.8%
Negative predictive values	78.4%	77.3%	78.9%	77.2%
Accuracy	80.5%	79.5%	81.1%	86.8%
κ-statistics	0.597	0.578	0.609	0.739
ρ	0.511	0.627	0.618	<0.0001
*OR (95% CI)	1.31 (0.66, 2.64)	1.24 (0.63, 2.45)	1.25 (0.62, 2.53)	0.09 (0.01, 0.40)

κ-Kappa statistics, *McNemar Chi square test was used

Table 5: Performance of mRDTs compared with PCR by parasite density

	Parasite Density		
	<200/μL	200-500/μL	>500/μL
Number of children (N)	6	4	79
Sensitivity (%)			
SD-Bioline™ versus PCR	100.0	100.0	97.5
Carestart™ versus PCR	75.0	100.0	97.5
ParaCheck™ versus PCR	100.0	100.0	100.0
SD-Bioline™ versus PCR			
True Positive, <i>n</i> (%)	4 (66.7)	4 (100.0)	77 (97.5)
False Positive <i>n</i> (%)	2 (33.3)	0	0
True Negative <i>n</i> (%)	0	0	0
False Negative <i>n</i> (%)	0	0	2 (2.5)
Carestart™ versus PCR			
True Positive, <i>n</i> (%)	3 (50.0)	4 (100.0)	77 (97.5)
False Positive <i>n</i> (%)	2 (33.3)	0	0
True Negative <i>n</i> (%)	0	0	0
False Negative <i>n</i> (%)	1 (16.7)	0	2 (2.5)
ParaCheck™ versus PCR			
True Positive, <i>n</i> (%)	4 (66.7)	4 (100.0)	77 (97.5)
False Positive <i>n</i> (%)	2 (33.3)	0	0
True Negative <i>n</i> (%)	0	0	0
False Negative <i>n</i> (%)	0	0	2 (2.5)

the form of Kappa statistics in Table 3. The odds of obtaining a positive test results were significantly higher when microscopy was used than those of SD-Bioline™ (OR = 13.5; 95% CI: 3.13, 82.0.9),

CareStart™ (OR = 9.0; 95% CI: 2.61, 37.19) or ParaCheck™ (OR = 13.0; 95% CI: 3.01, 79.9).

Using PCR as the gold standard, sensitivity and specificity for microscopy were 79.0% and 97.5%, respectively [Table 4]. For the three RDTs, the sensitivity was 85.5%, 84.6%, and 85.5% and specificity was 73.4%, 73.4% and 75.0% for SD-Bioline™, Carestart™, and Paracheck™, respectively. The PPV was 97.8%, 81.7%, 81.6%, and 82.9% for microscopy, SD-Bioline™, CareStart™, and ParaCheck™ respectively and the negative predictive value were 77.2%, 78.4%, 77.3% and 78.9% for microscopy, SD-Bioline™, Carestart™, and ParaCheck™, respectively. Out of the 190 samples, expert microscopy when compared with PCR showed that there were 87 (45.8%) true positive, 23 (12.1%) false negative, 2 (1.1%) false-positive and 78 (41.1%) true negative. Table 4 also shows that the diagnostic accuracy of the different test methods was 86.8% for expert microscopy, 80.5% for SD-Bioline™, 79.5% for CareStart™ and 81.1% for ParaCheck™ recorded 79.0%. The measure of agreement of the diagnostic methods yielded estimated Kappa statistics of 0.739 for microscopy and 0.597 for SD-Bioline™, 0.578 for CareStart™ and 0.609 for ParaCheck™ [Table 4]. The odds of PCR giving a positive test result compared with all the three mRDTs were not significantly different but significantly lower than expert microscopy (OR = 0.09; 95% CI: 0.01, 0.40).

The sensitivity of the three evaluated mRDTs compared to the PCR as the gold standard was shown in Table 5. All three mRDTs had a sensitivity of 100% when the parasite count was between 200 and 500 parasites/ μ L, had a sensitivity of less than 100 per cent at <200 parasites/ μ L, and only ParaCheckTM had a sensitivity of 100% when the parasite count was >500 parasites/ μ L. Details of the results of the mRDTs were also shown in Table 5. Notably, the results of the tests vary considerably between the three mRDTs when the parasite counts were <200 parasites/ μ L. However, when parasite counts were >500 parasites/ μ L, all three mRDTs had 97.5% true positive results, only 2.5% false-negative and no false positive and true negative results.

DISCUSSION

We evaluated the three most commonly used mRDTs in Nigeria that are on the WHO FIND list to guide healthcare practitioners and policymakers on the consistency or otherwise of the HRP2-based mRDTs (SD-BiolineTM, CarestartTM, and ParacheckTM) compared with microscopy and PCR as reference standards. This study revealed that the sensitivity of all three mRDTs, SD-BiolineTM, CareStartTM, and ParacheckTM were good using either microscopy or PCR in detecting malaria infection in febrile children. Similarly, all three mRDTs had high specificities above 70% using microscopy or PCR as the gold standard. Our study also demonstrated high positive predictive values and a high negative predictive value of expert malaria microscopy and mRDTs. However, microscopy gave higher sensitivity, specificity, positive and negative predictive values than the three mRDTs.

The prevalence of malaria by mRDTs and expert microscopy among febrile children was found to be 60% and 46.8%, respectively. There was a higher sensitivity for mRDT than for expert microscopy but lower specificity compared to PCR as a gold standard. The malaria prevalences by mRDTs, microscopy and PCR are considerably high in our study population compared with those reported by Ojurongbe *et al.*^[29] Sani *et al.*^[30] and Uzochukwu *et al.*^[31] but lower than those reported by Azikiwe *et al.*^[32] and Elechi *et al.*^[18] Our RDT positivity level, however, was remarkably more than that achieved in these studies,^[29-32] but it was higher than 2.3% achieved by Elechi *et al.* at Maiduguri.^[18] Since these studies^[18,29-32] used the HRP-2-based mRDT, the plausible explanations for the observed differences can be attributed to variations in the quality of RDT, sizes of the study population, and the technique used in the detection of malaria parasites. High malaria prevalence

implies that malaria remains a common and significant cause of fever in children under 5 years of age. The mRDTs remain a veritable tool to guide the treatment of malaria infection in primary health care. Also, it should be noted that the observed malaria prevalence in this study shows a reduction in the malaria infection rate among under-5 children in the study area from 77.6% (SD-Bioline RDT cassettes were re-read by an experienced clinical assistant) in the earlier study to 60.5% in this study. The observed reduction in malaria prevalence may be due to improvement in the distribution of long-lasting insecticide-treated nets (LLITNs) and use of ACTs advocated by the Oyo State Ministry of Health and other non-governmental organizations (NGOs) in the study area.

Specificity was lower in the mRDTs than expert microscopy which is common with false positive or false negative mRDT results. We recorded 1.1% false-negative results for SD-BiolineTM and ParaCheckTM and 1.6% for Carestart with parasite density ranging from range = 28–611,600/ μ L. When compared with expert microscopy, one of the false-negative sample recorded by all three mRDTs had parasite density of 5,538 parasites/ μ L. This may be attributable to either HRP-2 gene deletion or variation in the amino acid repeats in the HRP-2 gene sequence.^[33-35] It has been widely reported that HRP-2 antigen is known to persist in the blood beyond 2 weeks after asexual parasites have been cleared from previous episodes of malaria infection resulting in false-positive results.^[36] Other factors associated with false-positive mRDT outcomes include cross-reactivity with human antibodies, especially anti-HRP-2 immunoglobulin G and other infectious diseases.^[37,38] However, anti-malarial prescriptions for false-positive mRDT cases can be minimized by taking a thorough medical history from the patient and looking more inward for other causes of fever, especially if the patient has had recent malaria episodes and has been treated.

There was no significant difference in the performance of the individual evaluated mRDTs. The Kappa (*k*) value was 0.5 for the three mRDTs, indicating a moderate agreement with the gold standard. However, in comparison with the PCR, as calculated by the kappa coefficient, the agreement with expert microscopy was stronger than the mRDTs. This is a measure of consistency (good reliability).^[28] This finding may be closely related to the processes used by the tests to detect malaria infection. When mRDTs detect parasite antigens, the microscope performs a parasite morphological examination and the PCR detects whole blood parasite DNA.^[39] The importance of using microscopy in

clinical settings for the treatment of malaria cannot be overemphasized by these findings.

In this study, 97.5% (77/79) positive mRDTs were at parasite density >500 parasites/μL, we observed 100% (4/4) detection at 200-500 parasites/μL. Furthermore, SD-Bioline™ and ParaCheck™ had 100% (6/6) sensitivity at <200 parasites/μL while Carestart recorded 83.3% (5/6) when microscopy was used as the gold standard. Our data showed that the three mRDTs investigated fulfilled the requirement for its continued use in the population of the febrile child in the south-west of Nigeria. The WHO standard for RDT procurement for high transmission areas includes a recommendation that the panel detection scores against *P. falciparum* be at least 75% at 200 parasites/μL blood.^[22] This finding corroborates earlier reports from the same region of Nigeria. False-negative results from expert microscopy accounted for 11.6% (22 out of 190) compared to PCR results in this study. Quite often, at low parasitaemia (i.e. <200 parasites/μL), the performance of microscopy is lower than PCR.^[40,41] This has largely been attributed to the challenges of counting parasites at a low level of parasitaemia.

Malaria rapid diagnostic test kits targeting only HRP-2 antigen might fail to detect other species of human *Plasmodial* infections. In this study, PCR detected 8.4% of *P. malariae*, *P. ovale* 2.1% and co-infection of *P. falciparum*/*P. malariae* and *P. falciparum*/*P. ovale* accounted for 5.8% which the HRP-2 based mRDTs failed to detect. The consequence of this is the denial of ACTs to these sets of patients which if not properly followed up may lead to severe malaria that could lead to mortality as a result of such children being denied ACT based on negative mRDT results. Our result is consistent with earlier reports of *Plasmodium malariae* being responsible for about 7.5% of malaria infection in rural southwest Nigeria.^[42]

There are a few concerns that limit the interpretation of the findings of this study. Some of the patients might have been on anti-malarial treatment despite giving no history of their previous use of anti-malarial drugs to their parents or caregivers. That would have contributed to the false positive findings of RDT. The small sample size weakens the study's power. Thus, our finding could not be confidently generalised to the population of children in the south-west of Nigeria.

CONCLUSION

Based on the results of this study, the performance of the three HRP-2 based mRDTs is similar and quite good, indicating that any of the evaluated mRDT brands (SD Bioline™, Care Start™, and Paracheck™) could be

used for parasite-based diagnosis in case of management in the absence of microscopy. Given the existence of *P. malariae* and *P. ovale* in southwestern Nigeria, it may be necessary to review the policy of purchase of mRDT to include pan-specific mRDTs capable of detecting these species. As technology advances, super-sensitive and specific mRDTs are being designed that could negate the need to use pan-specific plus HRP-2 based mRDTs.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

There are no conflicts of interest.

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Additional file 1: Oligonucleotide pairs

Primer Name	Primer Sequence (5' to 3')	Reaction	Bp size	Target gene
rPLU5	CCTGTTGTTGCCTTAAACTT	First	20	18 SrRNA (<i>Plasmodium</i>)
rPLU6	TTAAAATTGTTGCAGTTAAAACG		23	
rFAL 1	TTAAACTGGTTTGGGAAACCAAATATATT		30	18 SrRNA (<i>P.falciparum</i>)
rFAL2	ACACAATGAACTCAATCATGACTACCCGTC	Nested	30	
Fw MAL 1	ATAACATAGTTGTACGTTAAGAATAACCGC		30	18 SrRNA (<i>P.malaria</i>)
Rv MAL 2	AAAATTCCCATGCATAAAAAATTATACAAA	Nested	30	
Fw OVA 1	ATCTCTTTTGCTATTTTTTAGTATTGGAGA		30	18 SrRNA (<i>P. ovale</i>)
Rv OVA 2	GGAAAAGGACACATTAATTGTATCCTAATG	Nested	30	