Evaluation of the Clinical Proficiency of RDTs, Microscopy and Nested PCR in the Diagnosis of Symptomatic Malaria in Ilorin, North-Central, Nigeria

Olalere Shittu[1*], Olufunke Adenike Opeyemi[1], Olusola Ajibaye[2], Babagbemi Olumuyiwa Omotesho[3], Oluwatosin Fakayode[4]

[1]*Parasitology Unit, Department of Zoology, University of Ilorin, Ilorin, Nigeria;* [2]*Biochemistry and Nutrition Unit, Nigeria Institute of Medical Research, Lagos, Nigeria;* [3]*University Health Centre, University of Ilorin, Nigeria;* [4]*Children Specialist Hospital, Centre-Igboro, Ilorin, Nigeria*

Abstract

BACKGROUND: Accurate laboratory diagnosis of suspected malaria is the hallmark to the control of the disease.

AIM: The clinical proficiency of commercial Rapid Diagnostic test kits (RDTs) using nested PCR as quality control was evaluated among patients attending two public healthcare providing institutions in Ilorin, Kwara state, North-Central, Nigeria.

METHOD: A cross-sectional evaluation of finger prick blood samples of volunteer patients were accessed for malaria parasites with pLDH, HRP2, Pf, Pf/PAN and nested PCR molecular assays. The data derived were analysed using standard formulae for diagnostic accuracy, and the obtained predictive values were subjected to a comparison with one-way analysis of variance (ANOVA).

RESULT: Three hundred and sixty-eight (368) patients comprising 203 (55%) females and 165 (45%) males participated in this study. Routine microscopy revealed that 54 (32.7%) males and 80 (39.4%) was infected with Plasmodium falciparum. SD Bioline (pLDH) 47.4%; Carestart Malaria (HRP2) 49.8% recorded low sensitivities. Micropoint (pfPAN) 82.8% and Micropoint (Mal. Pf) 64.4% recorded a high sensitivity. SD Bioline (pLDH) 67.4%; Carestart Malaria (HRP2) 85.9%; Micropoint (PfPAN) 62.2% and Micropoint (Mal. Pf) 86.7% had high specificities. The positive predictive value (PPV) ranged from 67.7% to 85.94%, while the negative predictive values (NPV) of 64.4% for SD Bioline (pLDH); 86.7% for Carestart Malaria (HRP2); 89.3% for Micropoint (pfPAN) and 58.5% for Micropoint (Mal. Pf). Agarose gel analysis of *P. falciparum ssrRNA* gene (206 bp) for 28 specimens containing 10% concordant and discordant samples showed that all 12 negative specimens for RDTs and routine microscopy were truly negative for nPCR. However, the remaining 16 specimens were positive for nPCR and showed discrepancies with routine microscopy and RDTs. Cohen's interrater diagnostic measure analysis revealed that the weighted kappa for the RDTs was moderate 0.417 (p=0.027), 95%CI (0.756, 0.078) and good for nPCR 0.720 (p < 0.001), 95%CI (0.963, 0.477). The area under the curve (AUC) specify that nPCR has been more effective than the RDTs (nPCRAUC = 0.875; p < 0.001 and RDTsAUC = 0.708; p = 0.063).

CONCLUSION: A thorough large-scale quality control is advocated on all commercial RDTs being used in most sub-Saharan African countries. This is to avoid double jeopardy consequent upon misdiagnosis on unidentified positive cases serving as pool reservoir for the insect vector and cyclical infection and re-infection of the populace.

Introduction

In spite of the continued efforts at eradicating malaria in sub-Sharan Africa, it has remained a major public health concern in the region. One of the remarkable milestones left to be achieved for the complete eradication of malaria is the development of adequate biological and clinical diagnostic tools [1] [2] [3] [4]. Microscopy has been the usual clinical practice for the diagnosis of malaria in endemic regions, but this method of diagnosis is faced with so many issues bothering on expertise, quality of field microscopy, epileptic electricity supply and poor reagents to mention a few [5] [6] [7] [8]. Although, detection thresholds of 4–20 parasites per microlitre are achievable using a Giemsa-stained thick blood film in controlled laboratory situations [9], thresholds of 100–

200 parasites per microlitre are more common in field settings [7] [8]. The use of rapid diagnostic tests as an alternative to microscopy in remote areas has however been advocated [1]. RDT was developed to improve the timeless sensitivity, and objectivity of malaria diagnosis through less reliance on expert microscopy [10]. These kits come in strips and cassettes embedded with immuno-chromatographic cork material that has antibodies targeted for *Plasmodium* parasite antigens. RDTs work on the detection of Histidine-Rich Protein2 (HRP-2) from *Plasmodium falciparum* and Parasite-Specific Lactate Dehydrogenase (pLDH) or *Plasmodium* aldolase from the parasite glycolytic pathway found in all species [11] [13]. RDTs are increasingly being used for malaria diagnosis because they are rapid and easier to use especially in limited resource settings and do not require trained personnel or special equipment [1] [8] [14] [15] [16]. One major pitfall in the use of RDTs in clinical situations is the issue of false-positive/false negative results which may lead to misdiagnosis and over administration of antimalarial drugs which often culminate into increased financial costs, side effects, and selection pressure for development of resistance in malaria parasite populations [17] [18] [19]. Inadvertently, false-negative results may lead to excess morbidity and mortality and further transmission. Thus, quality diagnostics are essential [20]. There are many commercial RDTs in the market today, but their efficacy remains a subject of concern. Therefore, it is not clear which RDT is more appropriate for different epidemiological settings [5] [21] [22]. It has been estimated that a diagnostic test with 95% sensitivity and 95% specificity requiring minimal infrastructure would avert more than 100,000 deaths and about 400 million unnecessary treatments (Long, 2009). The ominous disparity in RDTs and routine microscopy is gradually becoming contentious, hence the need for a more appropriate clinical diagnostic technique. A new diagnostic technique undergoing clinical trials becomes evident. Several molecular detection methods (MDM) are increasingly being researched in clinical practice [11] [23] [24] [25] [26]. It operates on the basis of small nuclear subunit ribosomal (SSU) rRNA genes which are targets, extensively used for the molecular detection of human malaria parasites [25]. These genes are known to have highly conserved regions and their copy numbers ranging from 4 to 8. These characteristics ensure that they are suitable genes for phylogenetic studies and molecular detection of *Plasmodium* parasites [27].

There are two basic approaches for species detection, single polymerase chain reaction and nested polymerase chain reaction (PCR). In general, nested-PCR is more sensitive than single PCR because they detect malaria parasites at very low-level malaria parasitemia [28]. The nested PCR is highly sensitive and has been widely used for diagnosis, confirmation of diagnosis, epidemiological studies, drug efficacy assessment and to measure the accuracy of microscopy [23] [29]. The nested PCR is used as a quality control laboratory technique to evaluate the clinical proficiency of established RDTs for this study. There is a dearth of published information on the assessment of the diagnostic efficiency of malaria in Ilorin, north-central Nigeria, thus the need for the present study. This study, therefore, seeks to comparatively evaluate the diagnostic efficiency of the various malaria diagnostic methods in Ilorin, north-central Nigeria.

Materials and Methods

A prospective study was conducted from January to March 2016 in Ilorin, Kwara State. A total of 368 subjects (203 males and 165 females) were enrolled at the following comprehensive health centres: University of Ilorin clinic and Children Specialist Hospital (Center Igboro) in Ilorin metropolis. The above areas were used to avoid bias in the sample collection, and so both children and adults were included in the study. The patients were screened for *Plasmodium falciparum* parasites using RDTs, Microscopy and nested PCR techniques.

Peripheral blood samples were collected from voluntary donors at the hospitals mentioned earlier. Examined samples were categorized into 2 groups; concordant and discordant samples after the methods of [24] with slight modifications. The concordant samples were those samples that tested simultaneously positive or negative with PLDH, HRP2 (RDTs) and Microscopy. The discordant group1 were samples that tested positive for PLDH, HRP2 but negative with Microscopy. The discordant groups 2 were samples that tested negative with PLDH, HRP2 but positive with microscopy. Dried blood spot (DBS) from all blood samples with discordant results and 5% of randomly selected samples with concordant results were analysed using nested PCR technique.

Patients of all age groups, presenting with signs and symptoms of malaria infections were included in the study. Demographic information, clinical details and basic information regarding prevention measures of the patients were recorded using questionnaires to establish inclusion and exclusion criteria. Venous blood samples were collected in EDTA and Plain bottles from the patients with suspected malaria cases. A drop was spotted on filter paper (Whatman no. 1). Each filter paper was dried at room temperature and stored carefully in a plastic container to avoid cross-contamination at -20ºC.

The diagnostic accuracies of the following RDTs were assessed, viz; *p*LDH (SD Bioline), HRP2 (Carestart Malaria), Pf/pan (Micropoint) and Mal. *Pf* (Micropoint). Fresh blood samples were transferred

directly from the EDTA bottle to RDTs sample pads with 2 drops of buffer solution (according to manufacturers' instruction). The buffer solution allows the blood to migrate towards the diagnostics and control line.

Thin and thick blood films were prepared following the methods of Gilles [30]. The slides were fixed in methanol in order to allow lysis of red blood cells. The slides were then stained with 10% Giemsa solution for 30 minutes, and thereafter washed, and screened under oil immersion (X 1000 Mg) Olympus microscope for *Plasmodium spp.* Parasite density was determined as the number of parasites per 200 leukocytes (WBC).

Dried blood spot (DBS) from all blood samples with discordant results and 5% of randomly selected samples with concordant results were analysed using molecular (nested PCR) technique. The various techniques were carried out in separate rooms.

Two methods were used for the DNA extraction according to Bereczky *et al.,* [31]. Briefly:

Tris-EDTA buffer-based extraction: Each filter paper punch was placed in Eppendorf tube, soaked in 65 μl of TE buffer and incubated at 50ºC for 15minutes. The punches were then pressed gently at the bottom of the tube several times, using pipette tip for each punch and heated at 97ºC for 15 minutes to elute the DNA templates. The liquid condensing on the lid and the wall of the tubes were removed by short centrifugation. DNA extract was kept at 4ºC for use within a few hours or stored at 20ºC.

Chelate extraction: Each filter paper punch was incubated overnight at 4ºC in 1ml of saponin in phosphate buffered saline (PBS). The punches were washed for 30 minutes in PBS at 4ºC, transferred into new tubes containing 25 μl of stock solution (20% chelex and 75 μl of distilled water) and vortexed for 30 seconds. The tubes were heated at 99ºC for 15 minutes to elute DNA templates.

A Master Mix containing all the reagents is prepared and aliquoted into the reaction tubes and overlaid with mineral oil. DNA templates generated from each sample by the two respective methods were added last into the master mix. Two amplification reactions were carried out. In the first amplification reaction (nest 1), a pair of oligonucleotide primers, which hybridised to a sequence in ssRNA gene of *Plasmodium falciparum*, were used. The product of the first reaction is then used as DNA template for a second amplification reaction (nest 2). The second amplification reaction involves the use of genus-specific and species-specific primers. The genus-specific and species-specific primers (oligonucleotide primers) was used to indicate the presence of malaria parasites and the *Plasmodium* species in the samples.

The PCR products from the amplification reaction were subjected to agarose gel electrophoresis. The electrophoresed products (result) were then interpreted by molecular detection specialist.

Data was entered and analysed using Statistical package for social sciences (SPSS 16.0) to determine the discrepancies between RDTs and nested PCR. The Sensitivity (sn), specificity (sp), positive predictive values (ppv) and negative predictive value (npv) were calculated using standard formulae, and their differences were analysed by comparing their mean values with One way ANOVA. The level of significance was estimated at $p < 0.05$. An interrater reliability analysis using the Kappa statistic was performed to determine consistency among raters. Cohen's interrater statistics was used to validate a measure of agreement between the diagnostic outcomes among the laboratory diagnostic tools.

Results

A Total of 368 patients; 203 (55%) females and 165 (45%) males were randomly enrolled and screened for malaria parasites using microscopy and RDT test kits. Four commercial RDTs were employed for this study, viz; Malaria Ag Pf/Pan by Standard Diagnostics Inc. Hagandong, Korea; Malaria HRP2 (Pf) by Carestart[TM] by Access Bio, Inc., New Jersey, USA; Malaria PF by Micropoint Inc. The USA.

From the PLDH (SD BIOLINE test kit), 145 (39%) were infected consisting of 72 (49.7%) males and 73 (50.3%) females. Carestart (HRP2) showed that 146 (39.7%) with male (72, 49.3%) and female (74, 50.7%). Micropoint (pfPAN) reported 167 (45.4%) with male (87, 52.1%) and female (80, 47.9%). Results from microscopy indicated that 234 (63.6%) were infected, consisting of 111 (47.4%) males and 123 (52.6%) females. However, only Micropoint (pfPAN) and (*Mal.* Pf) were statistically significant as regards sex at P > 0.05 (Table 1).

Table 1: Diagnostic detection of *P. falciparum* based on gender

Diagnostics	Prevalence of Malaria infections		
	Sex	Infected n (%)	Uninfected n (%)	p-value
SD Bioline (pLDH)	Male	72 (49.7)	93 (41.7)	0.082
	Female	73 (50.3)	130 (58.3)	
Carestart Malaria (HRP2)	Male	72 (49.3)	93 (41.9)	0.098
	Female	74 (50.7)	129 (50.1)	
Micropoint (pfPAN)	Male	87 (52.1)	78 (38.8)	0.007
	Female	80 (47.9)	123 (61.2)	
Micropoint (Mal. Pf)	Male	72 (46.8)	93 (43.5)	0.028
	Female	82 (53.2)	121 (56.5)	
Microscopy	Male	111 (47.4)	54 (32.7)	0.112
	Female	123 (52.6)	80 (39.4)	

The diagnostic detection of *P. falciparum* across age-groups in the study revealed that there were discrepancies in the diagnostic accuracy of the four RDTs when compared to routine microscopy. For instance at age ≤ 5yrs; routine microscopy identified varying parasitaemia with all the subjects at that age group being infected (40, 100%). However, SD Bioline identified only two (2, 5.0%) as positive for *P. falciparum*. At age-group 6-15yrs, the results were not comparable as slight differences occurred with all the diagnostic methods. There was a change in trend at age 16-25 yrs where it was observed microscopy detected more subjects as malaria positive (56, 62.9%) when compared with the other four RDTs (SD Bioline 15, 38.5%; Carestart 13, 33.3%; Micropoint pfPAN 24, 27.0%; Micropoint Pf 26, 29.2%). The diagnostic accuracy of the RDTs appear to positively pick up with increasing age of the subjects, for instance at age 36-45 yrs, there was no appreciable differences in the diagnostic outcome of the subjects (SD Bioline 31, 34.8%; Carestart 23, 25.8%; Micropoint pfPAN 23, 59.0%; Micropoint Pf 23, 59.0%; Microscopy 17, 43.6%). Malaria positive subjects declined with routine microscopy at age ≥ 46 yrs (9, 34.6%) while the RDTs showed more positive results within this age group. However, the diagnostic tests were all statistically significant at p < 0.001 (Table 2).

Table 2: Diagnostic detection of *P. falciparum* across age-group

Age-grp.	Diagnostics	Prevalence of Malaria infections		
		Infected n (%)	Uninfected n (%)	p-value
≤ 5 yrs				
	SD Bioline (pLDH)	2 (5.0)	38 (95.0)	
	Carestart Malaria (HRP2)	4 (10.0)	36 (90.0)	
	Micropoint (pfPAN)	9 (22.5)	31 (77.5)	
	Micropoint (Mal. Pf)	9 (22.5)	31 (77.5)	
	Microscopy	40 (100)	0	
6-15 yrs				
	SD Bioline (pLDH)	57 (65.5)	30 (34.5)	
	Carestart Malaria (HRP2)	65 (74.7)	22 (25.3)	
	Micropoint (pfPAN)	65 (74.7)	22 (25.3)	
	Micropoint (Mal. Pf)	62 (71.3)	25 (28.7)	
	Microscopy	78 (89.7)	9 (10.3)	
16-25 yrs				
	SD Bioline (pLDH)	31 (34.8)	58 (65.2)	
	Carestart Malaria (HRP2)	23 (25.8)	66 (74.2)	
	Micropoint (pfPAN)	24 (27.0)	65 (73.0)	
	Micropoint (Mal. Pf)	26 (29.2)	63 (70.8)	
	Microscopy	56 (62.9)	33 (37.1)	
26-35 yrs				
	SD Bioline (pLDH)	28 (32.2)	59 (67.8)	
	Carestart Malaria (HRP2)	29 (33.3)	58 (66.7)	
	Micropoint (pfPAN)	27 (31.0)	60 (69.0)	
	Micropoint (Mal. Pf)	27 (31.0)	60 (69.0)	
	Microscopy	34 (39.1)	53 (60.9)	
36-45 yrs				
	SD Bioline (pLDH)	15 (38.5)	24 (61.5)	
	Carestart Malaria (HRP2)	13 (33.3)	26 (66.7)	
	Micropoint (pfPAN)	23 (59.0)	16 (41.0)	
	Micropoint (Mal. Pf)	23 (59.0)	16 (41.0)	
	Microscopy	17 (43.6)	22 (56.4)	
≥46 yrs				
	SD Bioline (pLDH)	12 (46.2)	14 (53.8)	
	Carestart Malaria (HRP2)	12 (46.2)	14 (53.8)	
	Micropoint (pfPAN)	19 (73.1)	7 (26.9)	
	Micropoint (Mal. Pf)	19 (73.1)	7 (26.9)	
	Microscopy	9 (34.6)	17 (65.4)	
Total				
	SD Bioline (pLDH)	145 (39.4)	223 (60.6)	<0.001
	Carestart Malaria (HRP2)	146 (39.7)	222 (60.3)	<0.001
	Micropoint (pfPAN)	167 (45.4)	201 (54.6)	<0.001
	Micropoint (Mal. Pf)	166 (45.1)	202 (54.9)	<0.001
	Microscopy	234 (63.6)	134 (36.4)	<0.001

Routine microscopy was used as a gold standard against the four RDTs. There was low sensitivity for the first two diagnostic RDTs, viz; SD Bioline (pLDH) 47.4%; Carestart Malaria (HRP2) 49.8%. However, Micropoint (pfPAN) recorded a high sensitivity of 82.8%, and Micropoint (Mal. Pf) had 64.4%. The probability that a diagnostic will indicate the absence of malaria parasite among those without the disease is called specificity and in the present study, the specificity of SD Bioline (pLDH) 67.4%; Carestart Malaria (HRP2) 85.9%; Micropoint (PfPAN) 62.2% and Micropoint (Mal. Pf) 86.7%. PPV has determined the probability that the positive results were positive. In the following RDTs, the ppv ranged from 67.7% to 85.94%. The probability that the subjects truly does not have malaria reflected with NPV values of 64.3% for SD Bioline (pLDH); 86.7% for Carestart Malaria (HRP2); 89.3% for Micropoint (pfPAN) and 58.5% for Micropoint (Mal. Pf) (Table 3).

Table 3: Performance of RDTs using routine microscopy as the gold standard

Diagnostics	Sensitivity	Specificity	PPV	NPV
SD Bioline (pLDH)	0.4739	0.6739	0.7078	0.6438
Carestart Malaria (HRP2)	0.4979	0.8593	0.8593	0.8667
Micropoint (pfPAN)	0.8278	0.6223	0.6773	0.8929
Micropoint (Mal. Pf)	0.6438	0.8667	0.7905	0.5850

Keys: PPV=positive predictive value; NPV=negative predictive value.

Malaria sensitive groups were divided into the concordant groups and the discordant groups. The concordant group was further divided into Group 1: all True positives (i.e. positive for pLDH, HRP2 and microscopy) and Group 2: True negatives (i.e. negative for pLDH, HRP2 and microscopy). The discordant groups were also divided into two: Discordant group false positives (i.e. positive for pLDH and HRP2 and negative for microscopy), and the discordant group 2: false negatives (i.e. negative for pLDH and HRP2 and positive for microscopy). The clinical accuracy of the diagnostics was evaluated with nPCR by taking 10% each of Groups 1 & 2 and randomly picking 4 samples each from Groups I & II (Figure 1).
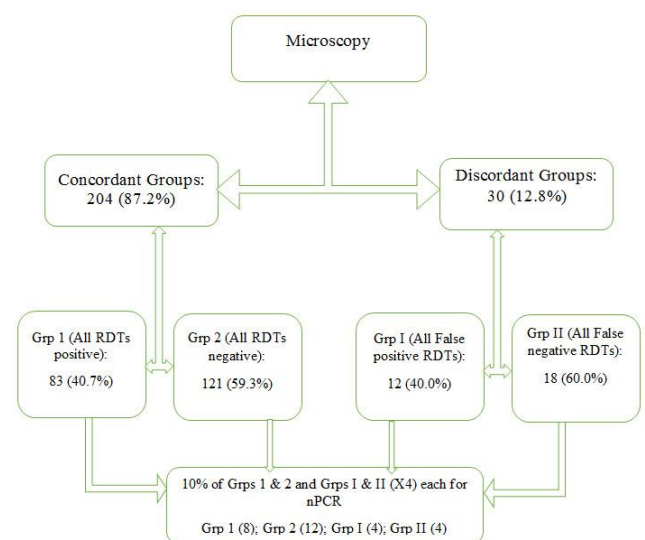

Figure 1: Flowchart describing the proportion of RDTs Sensitivity and samples subjected to nested PCR Analysis

The concordant group: Group 1: all True positives (i.e. positive for pLDH, HRP2 and microscopy) and Group 2: True negatives (i.e. negative for pLDH, HRP2 and microscopy). The discordant groups: Group 1: False positives (i.e. positive for pLDH and HRP2 and negative for microscopy), and Group 2: false negatives (i.e. negative for pLDH and HRP2 and positive for microscopy). The clinical accuracy of the diagnostics was evaluated with nPCR by taking 10% each of Groups 1 & 2 and 4 samples each from Groups I & II was randomly picked.

Agarose gel photograph of _P. falciparum_ ssrRNA gene (206 bp) resolved on 1.2% Agarose gel from 28 clinical samples of patients with malaria suspected cases provided the following results: Lanes 3, 5, 6, 8, 11, 13, 15, 19, 22, 24, 27 and 31 were PCR negative for _P. falciparum_. All the 12 specimens diagnosed and grouped as negative for RDTs and routine microscopy was truly negative for nPCR. This further shows that the RDTs specificity can be relied upon. Lane 1 and 17 = DNA Ladder while Lane 2 and 18 = 3D7 Positive control (Figure 2). However, lanes 4, 7, 9, 10, 12, 14, 16, 20, 21, 23, 25, 26, 28, 29, 30, 32 showed _P. falciparum_ positive cases meaning that the RDTs had low sensitivities as they initially reported non-malaria infections. To revalidate the present fallouts, the assays used as negative controls were subjected to nPCR and there was no amplification.
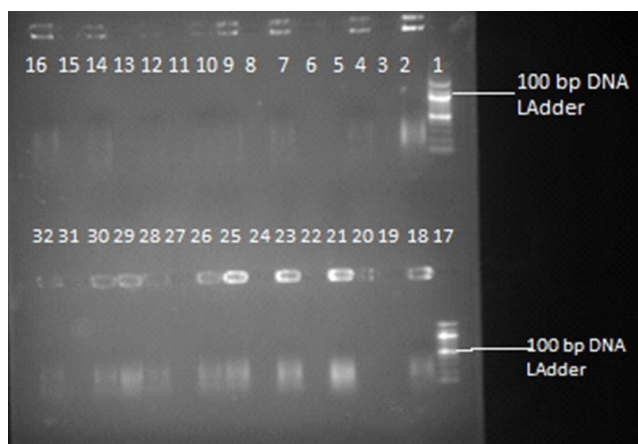

Figure 2: Schematic representation of Agarose gel photograph of P. falciparum ssrRNA gene (206 bp) from 28 clinical samples using species-specific oligonucleotide pairs for P. falciparum. Lanes 3, 5, 6, 8, 11, 13, 15, 19, 22, 24, 27 and 31 were PCR negative for P. falciparum. Lane 1 and 17 = DNA Ladder, while lane 2 and 18 = 3D7 Positive control. Lanes 4, 7, 9, 10, 12, 14, 16, 20, 21, 23, 25, 26, 28, 29, 30, 32 P. falciparum positive cases

Cohen's interrater diagnostic measure analysis revealed that the weighted kappa was estimated to assess the extent to which the sensitivity and specificity of RDTS and nPCR correspond with the routine microscopy in the diagnosis of malaria among a sampled population of the subjects. The kappa value for the RDTs was moderate 0.417 (p = 0.027), 95%CI (0.756, 0.078) and good for nPCR

0.720 (p < 0.001), 95%CI (0.963, 0.477). The area under the curve (AUC) is the percentage of randomly drawn pairs for which malaria is truly positive. The effectiveness of the RDTs and nPCR was also evaluated with AUC. The nPCR was found to be more effective than the RDTs (nPCR^{AUC} = 0.875; p < 0.001 and RDTs^{AUC} = 0.708; p = 0.063) (Table 4).

Table 4: Cohen's Interrater diagnostic measure for RDTs and nPCR with routine microscopy

Diagnostics	AUC	p-value	Kappa	95% CI Upper Bound	95% CI Lower Bound
Population for Quality control					
RDTs	0.708	0.027	0.417	0.756	0.078
nPCR	0.875	<0.001	0.720	0.963	0.477

Key: AUC: area under curve -0.5: worthless, to 1, a perfect test; Kappa < 0.20: poor, 0.41-0.60: moderate, 0.61-0.80: good and 0.81-1: very good; P is significant < 0.05. 95%CI: Estimate ± 1.96 SE.

Discussion

The diagnostic accuracy of malaria laboratory test reagents and kits has been a major pitfall in the efforts towards complete eradication of the disease in sub-Saharan Africa where presumptive evaluation has remained the norm [15] [16] [20]. The present study identified some incongruities between the diagnostic reliability of the sampled RDTs cum routine microscopy and nPCR. The diagnostic detectability of SD Bioline (pLDH) and Carestart Malaria

HRP2 were not gender significant (p > 0.05) while that of Micropoint (pfPAN) and (_Mal._ Pf) were statistically significant (p < 0.05). Concerning the stratified age groups in the study; a retinue of diagnostic discrepancies were also observed especially at age ≤ 5yrs, the RDTs failed to detect _Plasmodium falciparum_ antigens adequately. If the RDT results obtained for this age group were clinically relied upon; then diagnosis will be misleading the clinician. The low sensitivity recorded in the present situation among the ≤ 5 yrs group is in contrast with what was obtained in other studies [32] [33] [34] [35]. In previous studies, it was reported that RDTs failure to adequately indicate febrile illness usually occurs due to handling and storage [36] [37]. The RDTs on arrival in developing countries face challenges of immediate inspection and dispatch to health facilities and consequently are abandoned in the sun [38] [39].

The diagnostic accuracy picked up with increasing age in such a way as to believe that there exist a wider differences in addition to a substantial none overlap between the test methods. This assumption has also been reported by Endeshaw et al., [40]; Ojuroungbe et al., [15]; Osei-Yeboah et al., [16]. Although positive malaria subjects declined with routine microscopy (≥ 46yrs) which appears to be the normal occurrence in malaria endemic regions [41] [42] the results obtained with the sampled RDTs

_____

indicated an array of false negatives. The sensitivity of the RDTs (Pf/HRP2 and Pf PLDH) were low and fell below the threshold approved by WHO [1]. The observed low sensitivity in this study is similar to reports in other field studies [4] [43] [44] [45] [46]. A probable reason for these might be because some of the RDT kits rely on *Pf*HRP2 antigen for the detection of *P. falciparum*, and *Pf*HRP2 gene deletions often lead to false-negative results [43] [47] [48]. On the other hand, Baker *et al.,* [49] conducted studies on *Pf*HRP2 DNA obtained from isolates from African and South American countries with extreme sequence variation but concluded that diversity of the protein was not a major cause of the varying sensitivities of RDTs. Other possible explanations for the poor RDTs sensitivity, despite significant parasitemia, may include substandard diagnostics [50] [51], defects in device membrane [29], anti RDTs antibodies in human [7] and genetic diversity of parasite antigen *Pf*HRP2 and *Pf* PLDH [52].

Routine microscopy results were equivalent to those obtained by nPCR, except for few microscopically negative, but positive for nPCR as also reported by these authors [15] [22] [43] [53]. From a clinical perspective, failure to establish high parasite density from blood samples of febrile malaria patients can pose grave fatality [12] [20]. Diagnostic output with low specificity is less serious when compared with low sensitivity because presentations with low specificity only translate to overdiagnosis and an over the treatment of non- malaria cases [7] [44]. Faint colour bands formation on RDTs may also contribute to underreporting as subjective interpretation may contribute to poor performance [54]. This study used the nPCR techniques as a quality control technique between routine microscopy and available commercial RDTs. While appreciating that nPCR techniques provide astounding results even detecting parasitaemia at low microlitre, the high cost, complexity, materials and laborious exercise required may not make it a candidate diagnostic tool in many resource-poor communities [22] [55]. We determine the ability of a nested PCR assay to detect *Plasmodium* DNA in stored dried blood samples. The authors only choose the nPCR to ascertain the clinical diagnostic accuracy of the use of routine microscopy and RDTs in our settings. Often, malaria presentations at primary health providing centres in our communities are usually misleading, and the diagnostic failure of the RDTs may contribute over or/and under therapy.

Moreover, this has far-reaching implications such as treating patients on clinical suspicion, the threat of drug resistance, as well as possibility of missing a malaria case and consequent complications and mortalities [5] [36]. The WHO minimum selection criteria for malaria RDTs, i.e. panel detection score (PDS) against *P. falciparum* samples should be at least 75% at 200 parasites/µL [56]. WHO recommends that RDTs must demonstrate high sensitivity and specificity [1].

In conclusion, the results obtained from this study has indeed proven that DNA amplification technique provides a better diagnostic outcome as compared with microscopy and antigen-antibody complex detection. However, some of the shortcomings of the DNA amplification include; time, cost, sample quality and probably the volume of the sample available for PCR analysis. Furthermore, the ambiguity and complexity associated with the suitable choice of adequate RDTs to meet up with the prompt and immediate diagnosis are major challenges, especially now that the clinical approach to management, control, elimination and patient's well-being are paramount. Several factors may be militating against the reliability of any of these RDTs, some of these include genetic diversity of parasite antigen, parasite detection threshold, parasite target and level of malaria endemicity. A regular and thorough large-scale quality control is advised on all commercial RDTs imported into most sub-Saharan African countries, this is imperative to avoid the double jeopardy consequent upon misdiagnosis on unidentified positive cases serving as pool reservoir for *Anopheles* mosquitoes leading to cyclical infection and re-infection among the inhabitants.

Ethics approval and consent to participate

Authors' Contributions

OSH designed the protocols, performed the statistical analysis and wrote the final draft, OAO collected literature and wrote the first manuscript, OBO and OF provided volunteers for the research and obtained consent, OA carried out the nPCR analysis, OSH and OAO performed the routine microscopy, RDTs and administered the questionnaire.

Acknowledgements

_____

References

1. WHO. Guidelines for treatment of malaria. Geneva, Swithzerland: World Health Organization, 2006.

2. Perkins MDa, Bell DR. Working without a blindfold: The critical role of diagnostics in malaria control. Malaria Journal. 2008; 7:S5. https://doi.org/10.1186/1475-2875-7-S1-S5 PMid:19091039 PMCid:PMC2604880

3. D'Acremont V, Lengeler C, Mshinda H, Mtasiwa D, Tanner Ma, Genton B. Time to move from presumptive malaria treatment to laboratory-confirmed diagnosis and treatment in African children with fever. PLOS Medicine. 2009; 6:e252. https://doi.org/10.1371/journal.pmed.0050252 PMid:19127974 PMCid:PMC2613421

4. Mawili-Mboumba DP, Bouyou-Akotet MK, Mbouoronde CO, Kombila M. Analysis of malaria diagnosis discrepancies between RDTs and microscopy by nested PCR. J Biomed Sci & Engrg. 2013; 6:967-972. https://doi.org/10.4236/jbise.2013.610120

5. Faye B, Nath-Chowdhury M, Tine RC, Ndiaye JL, Sylla K, Camargo FW, Martel N, Foly K, Lo AC, Abiola A, et al. Accuracy of HRP2 RDT (Malaria Antigen P.f H) compared to microscopy and PCR for malaria diagnosis in Senegal. Pathogens and Global Health. 2013; 107(5):273-278. https://doi.org/10.1179/2047773213Y.0000000102 PMid:23916337 PMCid:PMC4001457

6. Tea BM. The distribution of Plasmodium falciparum infection durations. Epidemics. 2011; 3:109–118. https://doi.org/10.1016/j.epidem.2011.03.002 PMid:21624782

7. Bell D, Wongsrichanalai C, Barnwell JW. Ensuring quality and access for malaria diagnosis: how can it be achieved? Nature Rev Micro. 2006; 4:682–695. https://doi.org/10.1038/nrmicro1474 PMid:16912713

8. Slater HC, Ross A, Ouédraogo AL, White LJ, Nguon C, Walker PGT, Pengby Ngor RA, Silal SP, Dondorp AM, La Barre P, Burton R, Robert W, Sauerwein CD, Smith TA, Bousema T, Ghani AC. Assessing the impact of next-generation rapid diagnostic tests on Plasmodium falciparum malaria elimination strategies. Nature. 2015; 528:S94-S101. https://doi.org/10.1038/nature16040 PMid:26633771

9. Wongsrichanalai C, Barcus MJ, Muth S, Sutamihardja A, Wernsdorfer WH. A review of malaria diagnostic tools: microscopy and rapid diagnostic rest (RDT). Am J Trop Med Hyg. 2007; 77:119-127. PMid:18165483

10. Beadle C, Long GW, Weiss WR, et al. Diagnosis of malaria by detection of Plasmodium falciparum HRP-2 antigen with a rapid dipstick antigen-capture assay. Lancet. 1994; 343(8897):564–568. https://doi.org/10.1016/S0140-6736(94)91520-2

11. Abanyie FA, Arguin PMa, Gutman J. State of malaria diagnostic testing at clinical laboratories in the United States, 2010:

a nationwide survey. Malaria Journal. 2011; 10(1). https://doi.org/10.1186/1475-2875-10-340 PMid:22074250 PMCid:PMC3225402

12. Moody A. Rapid diagnostic tests for malaria parasites. Clinical Microbiology Reviews. 2002; 15(1):66-78. https://doi.org/10.1128/CMR.15.1.66-78.2002 PMid:11781267 PMCid:PMC118060

13. Tangpukdee N, Duangdee C, Wilairatana P, Krudsood S. Malaria Diagnosis: A Brief Review. Korean J Parasitol. 2009; 47(2):93-102. https://doi.org/10.3347/kjp.2009.47.2.93 PMid:19488414 PMCid:PMC2688806

14. McMorrow ML, Aidoo M, SP K. Malaria rapid diagnostic tests in elimination settings: can they find the last parasite? Clin Microbiol Infect. 2011; 17:1624–1631. https://doi.org/10.1111/j.1469-0691.2011.03639.x PMid:21910780 PMCid:PMC4821879

15. Ojurongbe O, Adegbosin OO, Taiwo SS, Alli OAT, Olowe OA, Ojurongbe TA, Bolaji OS, Adeyeba OA. Assessment of Clinical Diagnosis, Microscopy, Rapid Diagnostic Tests, and Polymerase Chain Reaction in the Diagnosis of Plasmodium falciparum in Nigeria. Malaria Research and Treatment. 2013; 2013:5. https://doi.org/10.1155/2013/308069 PMid:24371538 PMCid:PMC3859170

16. Osei-Yeboah J, Kwame Norgbe G, Yao Lokpo S, Khadijah Kinansua M, Nettey L, Allotey EA. Comparative Performance Evaluation of Routine Malaria Diagnosis at Ho Municipal Hospital. Journal of Parasitology Research. 2016; 2016:7. https://doi.org/10.1155/2016/5837890 PMid:27747097 PMCid:PMC5055952

17. Roper C, Pearce R, Nair S, Sharp B, Nosten F, Anderson T. Intercontinental spread of pyrimethamine-resistant malaria. Science. 2004; 305(5687):1124. https://doi.org/10.1126/science.1098876 PMid:15326348

18. Wellems TE, Plowe CV. Chloroquine-resistant malaria. J Infect Dis. 2001; 184(6):770-776. https://doi.org/10.1086/322858 PMid:11517439

19. White NJ, Pongtavornpinyo W, Maude RJ, Saralamba S, Aguas R, Stepniewska K, Lee SJ, Dondorp AM, White LJ, Day NP. Hyperparasitaemia and low dosing are an important source of anti-malarial drug resistance. Malar J. 2009; 8:253. https://doi.org/10.1186/1475-2875-8-253 PMid:19906307 PMCid:PMC2784792

20. Murphy SC, Shott JP, Parikh S, Etter P, Prescott WR, Stewart VA. Malaria Diagnostics in Clinical Trials. Am J Trop Med Hyg. 2013; 89(5):824-839. https://doi.org/10.4269/ajtmh.12-0675 PMid:24062484 PMCid:PMC3820323

21. Hopkins H, Bebell L, Kambale W, Dokomajilar C, Rosenthal PJa, Dorsey G. Rapid diagnostic tests for malaria at sites of varying transmission intensity in Uganda. The Journal of Infectious Diseases. 2008; 197(4):510–518. https://doi.org/10.1086/526502 PMid:18240951

22. Getnet G, Getie S, Srivastava M, Birhan W, Fola AA, Noedl H. Diagnostic performance of rapid diagnostic tests for the diagnosis of malaria at public health facilities in north-west Ethiopia. Tropical Medicine & International Health. 2015; 20(11):1564-1568. https://doi.org/10.1111/tmi.12570 PMid:26211505

23. Golassa L, Enweji N, Erko B, Aseffa A. Detection of a substantial number of submicroscopic Plasmodium falciparum infections by polymerase chain reaction: potential threat to malaria control and diagnosis in Ethiopia. Malar J. 2013; 12:352-359. https://doi.org/10.1186/1475-2875-12-352 PMid:24090230 PMCid:PMC3850638

24. Kipanga PN, Omondi D, Mireji P, Sawa P, Masiga DK, Villinger J. High-resolution melting analysis reveals low Plasmodium parasitaemia infections among microscopically negative febrile patients in western Kenya. Malar J. 2014; 13:429-434. https://doi.org/10.1186/1475-2875-13-429 PMid:25399409 PMCid:PMC4246433

25. Proux S, Suwanarusk R, Barends M, Zwang J, Price R, Leimanis M: Considerations on the use of nucleic acid based amplification for malaria parasite detection. Malar J. 2011; 10:323-

_____

329. https://doi.org/10.1186/1475-2875-10-323 PMid:22034851 PMCid:PMC3219859

26. Steenkeste N, Incardona S, Chy S, Duval L, Ekala MT, Lim P, Hewitt S, Sochantha T, Socheat D, Rogier C et al. Towards high-throughput molecular detection of Plasmodium: new approaches and molecular markers. Malar J. 2009; 8:86. https://doi.org/10.1186/1475-2875-8-86 PMid:19402894 PMCid:PMC2686730

27. Bronzan RN, Mc Morrow ML, Kachur SP. Diagnosis of malaria: Challenges for clinicians in endemic and non-endemic regions. Mol Diagn Ther. 2008; 12:299-306. https://doi.org/10.1007/BF03256295 PMid:18803428

28. Taylor BJ, Howell A, Martin KA, Manage DP, Gordy W, Campbell SD. A lab-on-chip for malaria diagnosis and surveillance. Malar J. 2014; 13:179-184. https://doi.org/10.1186/1475-2875-13-179 PMid:24885206 PMCid:PMC4029813

29. Biswas S, Tomar D, Rao DN: Investigation of the kinetics of histidine-rich protein 2 and of the antibody responses to this antigen, in a group of malaria patients from India. Ann Trop Med Parasitol. 2005; 99:553-562. https://doi.org/10.1179/136485905X51463 PMid:16156968

30. H.M. G: Diagnostic methods in malaria. In: Essential Malariology. Edited by Gilles HM, Warrell, D.A. Edward Arnold, (3rd) ed. edn. London: Bruce – Chwatts, 1993.

31. Bereczky S, Martensson A, Gil JP, Farnert A. Short Report: Rapid DNA extraction from archive blood spots on filter paper for genotyping of Plasmodium falciparum. The American Journal of Tropical Medicine and Hygiene. 2005; 72(3):249-251. PMid:15772315

32. Nkrumah B, Acquah SEK, Ibrahim Lea. Comparative evaluation of two rapid field tests for malaria diagnosis: Partec Rapid Malaria Test and Binax Now Malaria Rapid Diagnostic Test. BMC Infectious Diseases. 2011; 11(1):1 - 8. https://doi.org/10.1186/1471-2334-11-143 PMid:21605401 PMCid:PMC3118144

33. Ajumobi O, Sabitu K, Nguku P, Kwaga J, Ntadom G, Gitta S, Elizeus R, Oyibo W, Nsubuga P, Maire M et al. Performance of an HRP-2 Rapid Diagnostic Test in Nigerian Children Less Than 5 Years of Age. The American Journal of Tropical Medicine and Hygiene. 2015; 92(4):828-833. https://doi.org/10.4269/ajtmh.13-0558 PMid:25711608 PMCid:PMC4385782

34. Akinyi Okoth S, Abdallah JF, Ceron N, Adhin MR, Chandrabose J, Krishnalall K, Huber CS, Goldman IF, Macedo de Oliveira A, Barnwell JW et al. 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):e0126805. https://doi.org/10.1371/journal.pone.0126805 PMid:25978499 PMCid:PMC4433255

35. Azikiwe CCA, Ifezulike CC, Siminialayi IM, Amazu LU, Enye JC, Nwakwunite OE. A comparative laboratory diagnosis of malaria: microscopy versus rapid diagnostic test kits. Asian Pacific Journal of Tropical Biomedicine. 2012; 2(4):307-310. https://doi.org/10.1016/S2221-1691(12)60029-X

36. Berhane A, Russom M, Bahta I, Hagos F, Ghirmai M, Uqubay S. Rapid diagnostic tests failing to detect Plasmodium falciparum infections in Eritrea: an investigation of reported false negative RDT results. Malaria Journal. 2017; 16(1):105. https://doi.org/10.1186/s12936-017-1752-9 PMid:28264689 PMCid:PMC5339986

37. Kobayashi T, Gamboa D, Ndiaye D, Cui L, Sutton P, Vinetz JM. Malaria diagnosis across the International Centers of Excellence for Malaria Research: platforms, performance, and standardization. Am J Trop Med Hyg. 2015; 93. https://doi.org/10.4269/ajtmh.15-0004

38. Visser T, Daily J, Hotte N, Dolkart C, Cunningham J, Yadav P. Rapid diagnostic tests for malaria. Bulletin of the World Health Organization. 2015; 93:862-866. https://doi.org/10.2471/BLT.14.151167 PMid:26668438 PMCid:PMC4669726

39. Albertini A, Lee E, Coulibaly SO, Sleshi M, Faye B, Mationg ML. Malaria rapid diagnostic test transport and storage conditions in Burkina Faso, Senegal, Ethiopia and the Philippines. Malar J. 2012; 11. https://doi.org/10.1186/1475-2875-11-406

40. Endeshaw T, Gebre T, Ngondi J, Graves PM, Shargie EB, Ejigsemahu Y, Ayele B, Yohannes G, Teferi T, Messele A, Zerihun M. Evaluation of light microscopy and rapid diagnostic test for the detection of malaria under operational field conditions: a household survey in Ethiopia. Malaria journal. 2008; 7(1):118. https://doi.org/10.1186/1475-2875-7-118 PMid:18598344 PMCid:PMC2474640

41. Sadrizadeh B. Malaria in the world, in the eastern Mediterranean region and in Iran: Review article. In: WHO/EMRO Report, 2001.

42. Maltha J, Gillet P, Jacobs J. Malaria rapid diagnostic tests in endemic settings. Clin Microbiol Infect. 2013; 19(5):399-407. https://doi.org/10.1111/1469-0691.12151 PMid:23438048

43. Yan J, Li N, Wei X, Li P, Zhao Z, Wang L, Li S, Li X, Wang Y, Li S et al. Performance of two rapid diagnostic tests for malaria diagnosis at the China-Myanmar border area. Malaria Journal. 2013; 12(1):73. https://doi.org/10.1186/1475-2875-12-73 PMid:23433230 PMCid:PMC3599043

44. Kyabayinze DJ, Tibenderana JK, Odong GW, Rwakimari JB, Counihan H. Operational accuracy and comparative persistent antigenicity of HRP2 rapid diagnostic tests for Plamodium falciparum malaria in a hyperendemic region of Uganda. Malaria Journal. 2008; 7:221 - 231. https://doi.org/10.1186/1475-2875-7-221 PMid:18959777 PMCid:PMC2584069

45. Hopkins H, Kambale W, Kamya MR, Staedke SG, Dorsey G, Rosenthal PJ. Comparison of HRP2- and pLDH-based rapid diagnostic tests for malaria with longitudinal follow-up in Kampala, Uganda. American Journal of Tropical Medicine and Hygiene. 2007; 76:1992-1997.

46. Rubio JM, Buhigas I, Subirats M, Baquero M, Puente S, Benito A. Limited level of accuracy provided by available rapid diagnosis tests for malaria enhances the need for PCR-based reference laboratories. Journal of Clinical Microbiology. 2001; 39:2736-2737. https://doi.org/10.1128/JCM.39.7.2736-2737.2001 PMid:11427610 PMCid:PMC88226

47. Maltha J, Gamboa D, Bendezu J, Sanchez L, Cnops L, Gillet P, Jacobs J. Rapid Diagnostic Tests for Malaria Diagnosis in the Peruvian Amazon: Impact of pfhrp2 Gene Deletions and Cross-Reactions. PLOS One. 2012; 7(8):e43094. https://doi.org/10.1371/journal.pone.0043094 PMid:22952633 PMCid:PMC3429466

48. Gamboa D, Ho MF, Bendezu J, Torres K, Chiodini PL, Barnwell JW, Incardona S, Perkins M, Bell D, McCarthy J et al. A large proportion of P. falciparum isolates in the Amazon region of Peru lack pfhrp2 and pfhrp3: implications for malaria rapid diagnostic tests. PLoS One. 2010; 5(1):e8091. https://doi.org/10.1371/journal.pone.0008091 PMid:20111602 PMCid:PMC2810332

49. Baker J, Ho M, Pelecanos A, Gatton M, Chen N, Abdullah S, Albertini A, Ariey F, Barnwell J. Global sequence variation in the histidine-rich proteins 2 and 3 of Plasmodium falciparum: implications for the performance of malaria rapid diagnostic tests. Mal Journal. 2010; 9:129-137. https://doi.org/10.1186/1475-2875-9-129 PMid:20470441 PMCid:PMC2893195

50. Harvey SA, Incardona S, Martin N, Lussiana C, Streat E, Dolan S, Champouillon N, Kyabayinze DJ, Mugerwa R, Nakanwagi G et al. Quality issues with malaria rapid diagnostic test accessories and buffer packaging: findings from a 5-country private sector project in Africa. Malaria Journal. 2017; 16(1):160. https://doi.org/10.1186/s12936-017-1820-1 PMid:28427428 PMCid:PMC5397682

51. Barbe B, Gillet P, Beelaert G, Fransen K, Jacobs J: Assessment of desiccants and their instructions for use in rapid diagnostic tests. Malar J. 2012; 11.

52. Baker J, McCarthy J, Gatton M, Kyle DE, Belizario V, Luchavez J, Bell D, Cheng Q. Genetic Diversity of Plasmodium falciparum

_____

Histidine-Rich Protein 2 (PfHRP2) and Its Effect on the Performance of PfHRP2-Based Rapid Diagnostic Tests. The Journal of Infectious Diseases. 2005; 192(5):870-877. https://doi.org/10.1086/432010 PMid:16088837

53. Mawili-Mboumba DP, Bouyou-Akotet MK, Mbouoronde CO, Kombila M. Analysis of malaria diagnosis discrepancies between RDTs and microscopy by nested PCR. J Biomedical Science and Engineering. 2013; 6:967-972. https://doi.org/10.4236/jbise.2013.610120

54. Gatton ML, Rees-Channer RR, Glenn J, Barnwell JW, Cheng Q, Chiodini PL, Incardona S, González IJ, Cunningham J. Pan-Plasmodium band sensitivity for Plasmodium falciparum detection in combination malaria rapid diagnostic tests and implications for clinical management. Malaria Journal. 2015; 14(1):115. https://doi.org/10.1186/s12936-015-0629-z PMid:25889624

PMCid:PMC4371878

55. Bharti AR, Letendre SL, Patra KP, Vinetz JM, Smith DM. Malaria Diagnosis by a Polymerase Chain Reaction–Based Assay Using a Pooling Strategy. The American Journal of Tropical Medicine and Hygiene. 2009; 81(5):754-757. https://doi.org/10.4269/ajtmh.2009.09-0274 PMid:19861605 PMCid:PMC2770880

56. WHO. Good practices for selecting and procuring rapid diagnostic tests for malaria. In. Geneva: World Health Organization, 2011.