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# Comparative Diagnosis of Falciparum Malaria Infections by Microscopy, Two RDTs, and Nested PCR in the Three States of North-Western Nigeria

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**Abstract**
One of the most pronounced problems in controlling the morbidity and mortality caused by malaria is limited access to effective diagnosis. Microscopy remains the gold standard for malaria diagnosis, but it is labor intensive, requires significant skills and time. Thus, this study was conducted in search of any prompt, reliable, and good alternative of microscopy, when it is not available. A total of 540 patients in the three states of north-western Nigeria were prospectively enrolled to compare the performance of the Pf-HRP2 rapid diagnostic tests (RDTs), Pf/PAN-pLDH RDTs, and nested polymerase chain reaction (nPCR) with gold standard expert microscopy by 2x2 contingency tables, using standard formulae. For *P. falciparum* diagnosis, the sensitivity (Sn) and specificity (Sp) of Pf-HRP2 RDTs was 82% and 95%, while the Sn and Sp of Pf/PAN-pLDH RDTs (line 1) was 75% and 99%. Both Sn and Sp of nPCR were excellent with 98% and 100% respectively. The sensitivities of RDTs in this study were not optimal for *P. falciparum* diagnosis. Although, nPCR can be a good alternative of microscopy but the cost, qualitative nature and urgency of obtaining results with suspected malaria limits its use in routine clinical practice. Thus, microscopy should remain the diagnostic test of choice for malaria in this region.
**Key word:** falciparum malaria, microscopy, RDTs, nPCR, Nigeria

## 1. Introduction

Malaria is a severe and often rapidly fatal disease, with a high potential cost for health if diagnosis fails and an infection is missed (Bell *et al*. 2006). Malaria has remained a major public health problem in Nigeria, and is responsible for 30% childhood and 11% maternal mortality (FMOH 2005). It accounts for 300,000 deaths each year and about 60% of outpatient visits (FY 2011). Together Nigeria and the Democratic Republic of the Congo account for over 40% of the estimated total of malaria deaths globally (WHO 2012).

Proper management of malaria cases within the first 24 hours of onset is considered to be the best way to reduce its morbidity and mortality. This would be adequately achieved if most of the patients had access to laboratory facilities (Kamugisha *et al.*, 2008). Most victims of malaria still die, because the disease is not diagnosed in time by health workers (Uzochukwu *et al.* 2009).

In 2010, WHO recommended a universal "test and treat" strategy for malaria that has switched from symptom-based diagnosis to parasite-based testing of all fever cases (WHO 2010), but this has been preceded by a vigorous debate (Graz *et al*. 2011). This shift requires the availability of reliable diagnostic tests (Rosenthal 2012). Although, the percentage of reported suspected cases receiving a parasitological test has increased, from 67% globally in 2005 to 73% in 2009 (WHO 2011), but many cases still are treated presumptively without a parasitological diagnosis (WHO 2011).

Microscopy is considered to be the gold standard for malaria diagnosis (WHO 2004), but unfortunately, in many health facilities in sub-Saharan Africa there is a lack of properly functioning microscopes, quality control systems and well-trained laboratory technicians (Allen *et al*. 2011). Only a small proportion of all malaria diagnoses

 

in Nigeria are based on microscopy and the quality of those diagnoses is unknown (FY 2011).

Alternative methods have been studied in order to replace or complement the diagnosis through microscopy (DiSanti *et al*. 2004). PCR based on de-oxyribo nucleic acid (DNA) amplification have been applied to malaria diagnosis since the late 1980s (Rougemont *et al*. 2004). It is able to detect parasitaemias as low as 1–5 parasites/µl (Paris *et al*. 2007), but is laborious, costly, time consuming, and requires high technical experience (Tangpukdee *et al.* 2009). The introduction of malaria RDTs in the early 1990s ushered in a new era of parasite-based diagnosis that was expected to challenge the limitations of other diagnostic tests. The three antigens utilized in RDTs are *P. falciparum* Histidine rich protein 2 (Pf-HRP2), *Plasmodium* lactate dehydrogenase (pLDH) and aldolase (Murray *et al.* 2003).

Many variables can influence the performance of diagnostic tests in different settings. Therefore, wherever possible, test evaluations should be performed under the range of conditions, in which they are likely to be used in practice (Evaluating diagnostics 2006).

## 2. Materials and methods

### *2.1 Study area and study population*

A prospective field study was conducted from July to December 2011 in Sokoto, Kebbi, and Zamfara states, of north-western Nigeria. A total of 540 patients, 180 at each health center were enrolled at the Comprehensive Health Centre, Kware, Sokoto; General Hospital, Aliero, Kebbi; and General Hospital, Talata Mafara, Zamfara state. Patients of all age groups, presented with signs and symptoms of uncomplicated malaria with fever or the history of fever during the preceding 48 hours, had been included in the study. Patients presenting with severe malaria or any other severe illness and pregnant women were excluded. Demographic information, clinical details, and basic information regarding malaria prevention measures were recorded on a standardized questionnaire (Table 1). Venous blood samples were collected in EDTA bottles after obtaining informed consent from the adult participants and from guardians in the case of minors.

### *2.2 Ethical approval*

The study was approved by the ethical committee of Usmanu Danfodiyo University Teaching Hospital (UDUTH), Sokoto and permission was granted by the management of the health centers.

### *2.3 Malaria parasite test by microscopy*

Microscopic examination of thick blood smears was performed at the School of Medical Laboratory Science, UDUTH, Sokoto and used as gold standard. After staining with 3% Giemsa stain for 30 minutes, smears were read by experienced microscopist, who was blinded to the RDTs results. Smears were considered negative if no parasites were seen in 100 oil-immersion fields on a thick blood film. Parasites were counted against 200 white blood cells (WBC)/µl.

### *2.4 Pf-HRP2 RDTs*

For detecting *P. falciparum* malaria, using Paracheck Pf® dipsticks (Orchid Biomedical System, Goa, India) 5 µl fresh blood samples was transferred from EDTA bottles, directly to the sample pad and was placed into a clean test tube containing four drops of clearing buffer. Results were read as recommended by the manufacturers after 15 minutes (min), blinded to other diagnostic results.

### *2.5 Pf/PAN-pLDH RDTs*

In each CareStart™ 3-line Pf/PAN-pLDH RDTs (Access Bio Inc, New Jersey, USA) 5 µl of blood was delivered to the sample reception well. Two drops of buffer were then used to allow the blood to migrate towards the diagnostic and the control lines. Results were read after 20 min, blinded to other diagnostic results, according to the manufacturer's instructions.

### *2.6 Malaria parasite DNA extraction*

Nested PCR was performed at National Institute of Malaria Research, New Delhi, India. Parasite DNA was extracted from the same RDT strips (Veron & Crame, 2006 and Cnops *et al*. 2011), used for malaria diagnosis, using the QIAamp® DNA mini kit (QIAGEN, Hilden) according to manufacturer's protocol.

### *2.7 Nested polymerase chain reaction*

Amplification of 18S rRNA genes was done by nested PCR, using genus-specific (nest 1) and species-specific (nest 2) primers (Eurofins Genomics India Pvt. Ltd, Bangalore, India) as described by Snounou & Singh (2002) with some modifications according to Johnston *et al*. (2006).

Nest 1 PCR was carried out in a 20 µl reaction consisting of 2µl of 10X buffer, 2µl of $MgCl_2$, 2µl of dNTPs, 1µl of primer rPLU6 (forward),1µl of primer rPLU5 (reverse), 0.2µl of Taq polymerase enzyme, 2µl of sample DNA

 

and 9.8 µl of $ddH_2O$. Amplification was performed under the following conditions: Step 1: $94^0$C for 10 min; Step 2: $94^0$C for 1 min; Step 3: $60^0$C for 2 min; Step 4: $72^0$C for 2 min; Step 5: steps 2–4 was repeated for a total of 30 cycles; Step 6: $72^0$C for 5 min.

For nest 2 PCR, 2 µl of 10X buffer, 2 µl of $MgCl_2$, 2 µl of dNTPs, 1 µl of rFAL1 (forward) and 1µl of rFAL2 (reverse) primer specific for *P. falciparum* species, 0.2 µl of Taq polymerase enzyme, 2 µl of template DNA and 9.8 µl of $ddH_2O$ was used with a final reaction volume of 20 µl. Amplification was performed under the following conditions: Step 1: $94^0$C for 10 min; Step 2: $94^0$C for 1 min; Step 3: $55^0$C for 2 min; Step 4: $72^0$C for 2 min; Step 5: steps 2–4 was repeated for a total of 30 cycles; Step 6: $72^0$C for 5 min.

Nested PCR amplification product was detected via ethidium bromide, the stain used in 1.5% agarose gel electrophoresis. A positive reaction is noted when primers for *P. falciparum* produce amplification products of 205-bp.

***2.8 Data analysis***

Data was entered and analyzed using Epi-Info® (version 3.5.3). Both RDTs and PCR results was compared to expert microscopy, the gold standard, by using 2x2 contingency tables. From these, Sn, Sp, positive predictive value (PPV), and negative predictive value (NPV) were calculated, using standard formulae.

## 3. Results

***3.1 Baseline characteristic of study population***

Out of 540 patients enrolled in the study, there were 269 (49.8%) females and 271 (50.2%) male. Patients mean age was 20 years (SD ± 15.8, range 7 months to 80 years). Those under 5 years of age were 113 (20.9%) while 110 (20.4%) were aged between 5 and 14 years, with the remaining 317 (58.7%) were aged ≥15 years. The baseline characteristics of the study participants are shown in Table 1.

The most frequent symptom was fever or history of fever in 510 (94.4%) patients, followed by chills in 298 (67.7%), myalgia in 361 (66.9%), headache in 117 (21.6%), and vomiting in 210 (38.9%) patients.

***3.2 Expert microscopy results for P. falciparum diagnosis***

The 31 samples diagnosed as non-*P.falciparum* by Pf/PAN-pLDH based RDTs were excluded for analysis of *P. falciparum* malaria diagnosis. Three hundred and fifty nine (70.5%) patients out of 509 samples were diagnosed as malaria infected by *P. falciparum* by expert microscopy and one hundred and fifty (29.5%) samples were malaria parasite negative. Out of 359 malaria diagnosis, made by expert microscopy, 115 malaria diagnoses was made in Kware (Sokoto), 129 in Aliero (Kebbi), and 115 malaria diagnosis in Talata Mafara (Zamfara). Out of 150 negatives by expert microscopy, 53 negatives were in Kware, 41 negatives in Aliero, and 56 negatives were in Talata Mafara.

***3.3 Pf-HRP2 based RDTs versus gold standard expert microscopy***

Pf-HRP2 RDTs results were positive in 301 (59.1%) cases and negative in 208 (40.9%) out of 509 patients. Among the 359 positive cases diagnosed by expert microscopy, Pf-HRP2 RDTs result was true positive (by both expert microscopy and Pf-HRP2 RDTs) in 293 (81.6%) cases. Pf-HRP2 RDTs result failed to detected 66 (18.4%) cases, positive by microscopy, yielding 66 false negative results. Also, among the 150 negative cases detected by expert microscopy, 8(5.3%) cases were Pf-HRP2 positive RDTs result, yielding 8 false positive results and 142 (94.7%) cases were both Pf-HRP2 RDTs and expert microscopy negative (true negative). The Sn, Sp, PPV, and NPV of Pf-HRP2 RDTs were 82%, 95%, 97%, and 68% respectively (Table 2).

***3.4 Pf/PAN-pLDH based RDTs versus expert microscopy***

For *P. falciparum* analysis of Pf/PAN-pLDH based RDTs, there were 269 (52.8%) positive results and 240 (47.2%) negative results. The results of Pf/PAN-pLDH RDTs for *P. falciparum* (Line 1) were in agreement (true positive) in 268 (74.7%) cases and disagreement (false positive) in 91(25.3%) cases out of 359 positive diagnoses by expert microscopy. Out of 150 expert microscopy negative cases 149 (99.3%) were true negative (negative for both Pf/PAN-pLDH RDTs and expert microscopy) and 1 (0.7%) case was positive by Pf/PAN-pLDH RDTs, but negative by expert microscopy, thus making it false positive. The Sn, Sp, PPV, and NPV of Pf/PAN-pLDH RDTs were 75%, 99%, 100%, and 62% respectively (Table 2).

***3.5 Nested PCR versus expert microscopy***

Nested PCR was true positive in 61 (98.4%) out 62 microscopically *P. falciparum* positive samples. The only one (1.6%) discrepant sample was nPCR negative (false negative) for *P. falciparum* but positive by expert microscopy. All 12 microscopically negative samples were also negative by nPCR. The Sn, Sp, PPV, and NPV of nPCR were

 

98%, 100%, 100%, and 92% respectively (Table 2).

**4. Discussion**

The findings of this study showed that Pf-HRP2 RDTs had low sensitivity at both high and low parasite density (PD) while Pf/PAN-pLDH RDTs had low Sn at only low PD. Both RDTs had shown high Sp. The low Sn of Pf-HRP2 RDTs observed in the current studies was similar to what has been reported in other field studies conducted in Nigeria (Happi *et al*. 2004; VanderJagt *et al*. 2005; Tagbo & Henrietta 2007). There have been concordant reports of low Sn of Pf-HRP2 RDTs (Kamugisha *et al*. 2009; Sayang *et al*. 2009; Bendezu *et al*. 2010; Ishengoma *et al*. 2011) and Pf/PAN-pLDH RDTs (Huong *et al*. 2002; Mason *et al*. 2002; Albadr *et al*. 2011) from different settings.

There are some possible explanations why Pf-HRP2 tests were negative despite significant parasitemia: defects in the device membrane (Bell *et al*. 2006); failure of the parasite to express the antigen, due to deletion of the gene *Pfhrp2* (Gamboa *et al*. 2010; Koita *et al*. 2012); a prozone-like effect at high PD (Gillet *et al*. 2009; Gillet *et al*. 2011; Luchavez *et al*. 2011); anti-Pf-HRP2 antibodies in humans (Biswas *et al*. 2005); differences in the transcription level of *pfhrp2* gene and expression of Pf-HRP2 antigen levels between different *P. falciparum* strains (Baker *et al*. 2011); a variant Pf-HRP2 antigen, not captured by the monoclonal antibodies (Lee *et al*. 2006); genetic diversity of the parasite antigen Pf-HRP2 (Baker *et al*. 2005). Pf-HRP2 antigen may have been internalized into the digestive vacuole in some strains rather than released into the bloodstream (Howard *et al*. 1986). It could also be explained by storage conditions (WHO 2003). However, in this study storage, procedures and transport were accordingly to manufactures instructions.

Several factors could have explained the low Sn of the Pf/PAN-pLDH RDTs: decreased pLDH activity with antimalarial therapy may have resulted in remnant non-viable and non pLDH producing (Iqbal *et al*. 2003), but parasites may be seen on blood smears (Mason *et al*. 2002); host metabolic and/or immune factors that could reduce target antigens or interfere with their binding to detecting antibodies (Moody *et al*. 2000). Antigen pLDH are generally thought to be less heterogeneous than HRP2 (Baker *et al*. 2005) but a small amount of genetic variation has been detected by Mariette *et al*. (2008).

High Sp observed in this study is in concordance with Paris *et al*. (2007); Ishengoma *et al*. (2011). The replacement of IgG with IgM can be the possible causes of high specificity of Pf-HRP2 RDTs. IgG antibody used to give higher rates of false positivity than IgM antibody, coated onto the strips (Mishra *et al*. 1999) because IgM are pre-absorbed against rheumatoid factor (RF) that neutralize the cross-reactivity of RF present in the blood (Swarthout *et al*. 2007). The use of artemisinin-compound artemether as first line treatment by doctors, in this setting, as recommended by National Antimalarial Treatment Policy of Nigeria can be the other possible cause because Singh & Shukla (2002) found a shorter time of clearance of Pf-HRP2 antigen after treatment with the it than other treatments. The excellent specificity of the Pf/PAN-pLDH RDTs in this study could be as a result of the advantage of the pLDH clearance after anti-malarial treatment (Moody 2002).

Nested PCR results were equivalent to those obtained by microscopy except one microscopically positive, but negative by nPCR, concordant with Coleman *et al*. 2006. According to Batwala *et al*. (2010) possible cause of this failure can be absence of the target sequence homologous to the oligonucleotide primers, may be due to deletion/mutation of sequence, or maybe it is present but inaccessible due to inhibition of nPCR by sample components or degradation of DNA during sample preparation and storage. Also, target DNA may not be accessible because of inadequate cellular lysis, or the target sequence copy number may be too low for amplicons to be detected under conditions used.

There are drawbacks of nPCR that limits its use as routine diagnostic method. It is cumbersome, expensive, time consuming, and unavailable in developing countries because of limited resources such as finance, electricity and inadequate laboratory infrastructure. Positive nPCR results cannot be directly correlated with a severity of infection because it cannot give a measure of parasite density. Therefore, nPCR would be preferred as an adjunct to microscopy for the confirmation of the clinical suspicion of malaria. Because nPCR is not affected by the subjectivity of the observer it can be introduce in reference laboratories for testing of samples from routine diagnostic laboratories to provide an excellent quality control and accurate epidemiological data.

From a clinical perspective, failure to diagnose malaria parasite at high PD is a serious cause of concern, as infection with *P. falciparum* is potentially fatal in the absence of appropriate treatment. Having a relatively low Sp is less serious than having a low Sn because this can only leads to an over-diagnosis and to an over-treatment of non-malaria cases.

 

**5. Conclusion**

According to the WHO recommendations, RDTs should demonstrate a minimum Sn of 95% (100% when parasite density is above 100/µl), and a minimum Sp of 90% (WHO 2006). The low Sn of RDTs in this study indicates that both RDTs did not perform well enough to be recommended as a diagnostic test for malaria diagnosis in this region, even though it was rapid, portable, easy to learn and use. Thus, our study emphasize that microscopy should remain the diagnostic test of choice for malaria diagnosis. The accuracy and utility of RDTs require further investigation of possible cause of low Sn of RDTs before the large scale implementation of malaria RDTs in this setting. Nested PCR can be a good alternative of microscopy but the cost, and urgency of obtaining results quickly for patients with suspected malaria limits the usefulness of nPCR in routine clinical practice.

Although, the RDTs Sn limitations remain hindrances to these assays, it can be used in certain circumstances, such as when microscopy services are not operational (e.g., power failure, evenings, weekends, and public holidays) or as a primary diagnostic tool for rural/ remote areas without microscopy services, but should not be regarded as a first-line diagnostic test.

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**Table 1: Baseline characteristics of the study population**

| Characteristics | All site | Aliero | Kware | Talata Mafara |
|---|---|---|---|---|
| Number of subjects | 540 | 180 | 180 | 180 |
| Mean age in years ±SD | 20.2 ±15.8 | 17.9 ±17.0 | 21.9±14.1 | 20.8 ±16.0 |
| Number of male (%) | 271 (50.2%) | 93 (51.7%) | 86 (47.8%) | 92 (51.1% ) |
| Number of female (%) | 269 (49.8%) | 87 (48.3%) | 94 (52.2%) | 88 (48.9%) |
| Mean ±SD axillary temperature $^0$C | 37.0 ±1.06 | 37.0 ±1.1 | 36.9 ±1.0 | 37.0 ±1.0 |
| Always sleeps under a mosquito net | 258 (47.8%) | 85 (47.2%) | 86 (47.8%) | 87 (48.3%) |
| Insecticide Spray/ Mosquito coil | 221 (41%) | 91 (50.6%) | 73 (40.6%) | 57 (31.7%) |

**Table 2: Validity of diagnostic tests, compared to expert microscopy**

| Diagnostic Tests | | 2x2 Contingency table MP +ve | MP -ve | Sn (%) | Sp (%) | PPV (%) | NPV (%) |
|---|---|---|---|---|---|---|---|
| **Pf-HRP2 RDTs** | **+ve** | 293 | 8 | 82 | 95 | 97 | 68 |
| | **- ve** | 66 | 142 | | | | |
| **Pf/PAN-pLDH RDTs** | **+ve** | 268 | 1 | 75 | 99 | 100 | 62 |
| | **- ve** | 91 | 149 | | | | |
| **PCR** | **+ve** | 61 | 0 | 98 | 100 | 100 | 92 |
| | **- ve** | 1 | 12 | | | | |

*Key: Sn- Sensitivity, Sp- Specificity, PPV- Positive Predictive Value, NPV- Negative Predictive value*

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