Laboratory diagnosis of malaria: Comparing giemsa stained thick blood films with rapid diagnostic test (RDT) in an endemic setting in North-west Nigeria

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Malaria microscopy will continue to remain the gold standard for the diagnosis of malaria, but prolonged turnaround time and lack of malaria microscopist that are associated with this technique continue to impact on the quality of service rendered by the laboratory, especially the turnaround time, which is one of the quality indicators to measure laboratory quality systems. The prolonged turnaround time and lack of malaria microscopists identified with resource constraint setting in the midst of a period with high transmission of malaria prompted us to search for an alternative to the gold standard. Hence, multi-species were evaluated based on the rapid diagnostic test to determine its importance in the laboratory diagnosis of malaria. The rapid diagnostic test kit (SD Bioline – FK80) used in this study is based on the detection of Plasmodium falciparum, histidine rich protein-2 (HRP-2) and Plasmodium vivax-specific lactose dehydrogenase (Pv-pLDH), and was compared against traditional malaria microscopy. The evaluation was designed to provide data on the use of rapid diagnostic test and microscopy for malaria diagnosis in an urban secondary health facility with laboratory component. In total, 939 patients that consulted the out-patient department were clinically evaluated and suspected for malaria. Blood samples of those patients were subsequently tested using both techniques. The sensitivity and specificity for P. falciparum were 75.2 and 80.4%, respectively. The accuracy of the test was 76.8%. It was concluded that the rapid diagnostic test could be used as the first screening test for malaria diagnosis and have to be confirmed by microscopy if the outcome is negative. This will therefore improve on the current situation of treating patients for malaria without laboratory outcome to confirm clinical evaluation.

Key words: Rapid diagnostic kit, malaria, Nigeria, microscopy, sensitivity, specificity.

INTRODUCTION

The use of malaria rapid diagnostic tests (RDT) is becoming relevant considering its importance in malaria diagnosis in the absence of reliable microscopy. Though, microscopy remains the gold standard, it is usually labour intensive and requires well trained and skilled personnel (Moody, 2002; Murray et al., 2008). Even when trained and experienced microscopists are present, the microscope, its associated consumables and stable energy supply must be available. Poor or no availability to reliable microscopy have led to the use of RDT at almost every level of health care system (Murray et al., 2008). In
addition, some countries have evaluated the performance of different RDT kits for the purpose of product selection for their malaria control program (Chinkhumba et al., 2010; Gitonga et al., 2010; Mbonye et al., 2010; Singh et al., 2010). The proliferation of malaria RDT and constant evaluation of the RDT kits to determine its suitability in different regions and settings prompted the World Health Organization (WHO) to evaluate the malaria kits in 1998 and 1999 (WHO, 2009; 2010a) and its usage subsequently formed part of the WHO guidelines on malaria treatment in 2010 (WHO, 2010b).

However, the use of RDT for the diagnosis of malaria still comes with caution in certain endemic areas like Nigeria, due to its cost (N217/$1.50) on patients as compared to microscopy (N50/$0.30) and lack of published malaria RDT validation or evaluation data that is laboratory based in such areas.

In a typical government owned urban secondary health facility in an endemic setting like Nigeria, the work load on the laboratory is high due to the demand for malarial microscopy besides other tests. Usually, there is no dedicated microscope for malaria testing, over-worked microscopists and unstable supply of energy source to power the microscope. These shortcomings have significantly increased the turnaround-time (TAT) of malaria testing. Usually, the results become available for the clinician the next day while the recommended TAT is 2 h (WHO, 2010b). This leads to prescription of anti-malarial drugs by the clinicians irrespective of the outcome of malarial testing from the laboratory. In such cases, the clinicians do not make use of the laboratory result, a practice that is against the National and WHO treatment guidelines (Federal Republic of Nigeria, 2005; WHO, 2010b).

This study therefore explore another alternative to malaria testing by comparing *Plasmodium falciparum*, histidine rich protein-2 (Pi-HRP-2)/*Plasmodium vivax* specific lactose dehydrogenase (Pv-pLDH) based RDT (SD Bioline, FK80) with microscopy in an urban secondary health facility. The choice of this RDT was based on published data on its suitability for the detection of malaria antigens, Pi-HRP-2 and Pv-pLDH (Murray et al., 2008; Gillet et al., 2009) and WHO evaluation outcome (WHO, 2010a). *P. falciparum* and *P. vivax* co-infection observed in the studied area from experience using the light microscope, also informed the decision of three band malaria RDT used in this study.

**MATERIALS AND METHODS**

**Study setting**

This study was conducted in a secondary level health facility located in Sokoto, in the North-west of Nigeria. The geographical location is latitude 13.05’273” and longitude 05.24’615”. The hospital serves the population within the capital in addition to a tertiary hospital and private hospitals. However, the majority of residents access the secondary facility because of the low cost of treatment as compared to other hospitals within the capital. Malaria transmission in Sokoto is perennial, and peaks from June to August and from October to December, after the seasonal rains.

**Study design and laboratory techniques**

This study was undertaken between October and December, 2010, patients included in the study were individuals attending the outpatient department of the hospital. They were clinically evaluated and patients suspected for malaria were referred to the laboratory for malaria diagnosis by microscopy and RDT analysis after a verbal consent to participate in the study. Excluded were individuals below the age of 5 years and pregnant women. A total of 939 patients suspected for malaria were recruited.

**Patients’ sample**

Four milliliter blood was collected in an ethylenediaminetetraacetic acid (EDTA) container because of other associated tests like widal and full blood count requested by the clinician. Hence, a drop of the blood was used to prepare thick film, while a drop of plasma was used for RDT after centrifugation at 1,500 rpm for 10 min. Plasma was used because test requisition from the clinician also included other tests as earlier mentioned.

**Standard microscopy**

*Preparation and detection of *P. falciparum* using thick film*

The air dried thick film was stained in 10% Giemsa solution for 15 min and subsequently read by three trained and skilled readers with a minimum experience of eight years on malaria microscopy. All the slides were initially read by 1st and 2nd reader. Discrepant results between these readers were resolved by the 3rd reader (the most experienced among the readers). 10% of negative slides were also confirmed by the 3rd reader. All the readers were blinded to their individual readings, patient’s clinical status, and RDT observer’s interpretations. The parasite density was determined on all positive slides by the 2nd reader and was corrected by the 3rd reader. The number of asexual parasites per 200 white blood cells, or 500 white blood cells for low density infections, was used to calculate the number of asexual parasites per microliter of blood. However, a standard count of 8,000 white blood cells per microliter of blood was assumed (WHO, 1991).

*Preparation and identification of *P. vivax* using thin film*

The air dried thin film was fixed in methanol and stained in 10% Giemsa solution for 15 min. This procedure was performed in order to identify and confirm *P. vivax* from a co-infection with *P. falciparum* using the RDT, but the density of *P. vivax* was not determined.

**RDT test platform**

The FK80 used is based on lateral flow immunochromatographic RDT in a cassette format. The cassette had three lines; a control line which indicated the validity of the test, a HRP-2 line that showed an infection with *P. falciparum*, and a single Pv-pLDH line that indicated an infection with *P. vivax*. The combination of both lines indicated a mixed infection with *P. falciparum* and *P. vivax*. The absence of all infection lines with the presence of control line...
only indicated no infection with neither of the parasites. The test kits had lot number of 018106 with an expiration date of 24th February, 2012.

**Antigen detection**

RDT that is able to simultaneously detect *P. falciparum* and *P. vivax* (SD Bioline-Malaria P.f/P.v, Standard Diagnostics Inc, Korea) was performed according to the manufacturer’s instructions. The readings were carried out at daylight and subsequently performed by two observers; the first observer read the test results after 20 min and the second observer confirmed the result at 30 min, still within the reading time mentioned by the manufacturer’s instructions (20 to 30 min). One positive test line was recorded as positive for either *P. falciparum* or *P. vivax*, two positive lines was captured as *P. falciparum* and *P. vivax* and the non-detection of both test lines with control line only was recorded as negative (Figure 1). The detection of any of the test lines without the control line was considered invalid.

In case of discordant results between the first and second observer as per test line intensities, the first observer reading was considered. The observers were blinded to patient’s clinical status and microscopy results.

**Data collection and statistical analysis**

The results of the microscopy and RDT were separately recorded on different worksheets and matched by an independent expert. After entering the data, the database was further verified by cross checking with the results in the worksheets for consistency, and errors identified were corrected. Data were then analyzed using Statistical Package for Social Sciences (SPSS) version 17 (Illinois, Chicago).

Sensitivity, specificity, positive and negative predictive value were calculated for *P. falciparum*, while sensitivity and positive predictive value only was calculated for *P. vivax*. All the indicators were determined using 95% confidence intervals (CI).

**Ethical approval**

This study protocol was approved by the Ethical Committee of The Specialist Hospital, Sokoto.

**RESULTS**

This study recruited 939 patients of age 5 to 74 years (mean age 20.2 ± 16.1) of which 644 were males and 295 were females. All recruited patients were tested using microscopy and SD Bioline RDT (Figure 2). A total of 663 (70.6%) were found to be positive by microscopy and 54.6% by RDT. It should be noted that mixed infections accounted for 3.6% by RDT and this was confirmed by microscopy except for one patient in which only *P. falciparum* parasites were seen.

The sensitivity and specificity of *P. falciparum* for RDT analysis was 75.2 and 80.4%, respectively. The positive predictive value was 90.2% and the negative predictive value was 57.5% (Table 1). The overall accuracy for the detection of *P. falciparum* by RDT was 76.8%. The sensitivity of *P. vivax* by RDT was 100%. The parasite densities of *P. falciparum* were grouped into seven categories as shown in Table 2. The sensitivity for detection of *P. falciparum* by RDT was as high as 95.9% for the group one with the lowest parasite density. It should be noted that this study was conducted in a high transmission area, hence, high density was recorded. Interestingly, groups 2 to 5 and 7 recorded low sensitivity.

**DISCUSSION**

It is expected that an appropriate malaria RDT should have high sensitivity (95%) and specificity (97%), and ability to detect low parasite density infections (WHO, 2003). This is contrary to this study, since the sensitivity (75.2%) and specificity (80.4%) of the product used was low as per *P. falciparum* diagnosis. Also, it has been
reported that the performance of FK80 can best be compared with other malaria RDTs by considering the antigens, HRP-2 and Pv-pLDH separately (Gillet et al., 2009). For the HRP-2 test line in the diagnosis of *P. falciparum* in a relative endemic setting, the sensitivities and specificities reported were in the range of 84 to 97% and 39 to 93%, respectively (Ashton et al., 2010; Chinkhumba et al., 2010; Singh et al., 2010). The specificity of HRP-2 as reported in this study is in agreement with other studies except for the WHO recommendation. This loss in specificity as per WHO recommendation can however be as a result of self-treatment,
because in our setting most patients treat themselves for malaria before presenting for care to the health facility. This self-treatment might have cleared their parasitaemia, but have residual circulating HRP-2 antigens (Chinkhumba et al., 2010).

The low sensitivity recorded indicates a high rate of false negative results which was not expected. It is also expected that the SD Bioline used in this case should detect high parasite densities (P. falciparum HRP-2) as reported in this study, but it is different. Since the minimum requirement for the detection of parasite density is 100 parasites/µl according to the WHO recommendation. There are studies that have attempted to explain false negative results; these studies associated gene deletions (Gamboa et al., 2010), variation in antigen structure (Baker et al., 2005; Lee et al., 2006) and prozone effect (Gillet et al., 2009; Luchavez et al., 2011) as responsible. The high false negative results recorded in this study are not acceptable since it have the potential for harming patient health and damaging the credibility of malaria control programmes. Also, failure to detect malaria parasitaemia could prevent a clinician from prescribing anti-malarial therapy. It is therefore important for the laboratorians to understand the likely causes of false negative results in malaria testing using malaria RDT, especially in an endemic setting like ours.

Studies have clearly demonstrated the impact of prozone effect on malaria RDT (Gillet et al., 2009; Luchavez et al., 2011). The prozone phenomenon (high dose hook effect) is a well-recognized limitation of assays that is based on antigen-antibody interactions like the one in this study. It is therefore possible that the high parasite densities recorded in this study prevented the activation of antigen-antibody complex which is required for the test lines to be visible. This effect has been specifically linked with P. falciparum HRP-2 (Gillet et al., 2009).

However, the high positive predictive value (90.2%) recorded is higher than previous studies that was in the range of 64 to 71% (Ashton et al., 2010; Singh et al., 2010) and this outcome provides an argument to use the RDT as screening as the platform consider patients with a positive result as truly malaria infected patients and the clinicians can therefore proceed with the treatment. It can also be argued that in a highly endemic setting, the first treatment to be considered for most cases presented at the out-patient department is malaria treatment. So, the risk of missing an individual infected with malaria may not be acceptable, otherwise it can be fatal.

The specificity of the test reported falls within the acceptable range when compared with previous studies as earlier mentioned and may allow us to diagnose patients as truly negative for falciparum malaria, but the low negative predictive value (57.5%) reported does not agree with previous studies in the range of 90 to 98% (Ashton et al., 2010; Singh et al., 2010), and this provided the risk of missing an infected individual. This outcome as per positive predictive value and negative predictive value therefore permitted us to propose malaria testing algorithm (Figure 3) for use in the laboratory especially in a secondary health facility like ours. According to this proposal, negative results as determined by SD Bioline RDT must be confirmed by microscopy before communication to the clinician, while positive results by RDT can be reported without confirmation by microscopy. This malaria serial testing algorithm will obviously meet the required TAT and reduce competition for the microscopy for other laboratory tests while at the same time provide an accurate and reliable result to the clinicians. It is of note that this proposal does not exclude further search of other RDT formats or brands.

The diagnosis of P. vivax is serious and has high transmissibility; hence, it requires specific therapy (Gillet et al., 2009). The SD Bioline used was able to detect PVP-LDH and its sensitivity (97.1%) recorded is higher than Gillet et al. (2009) study (75.8%). Also, this study was able to detect mixed infection which makes FK80 suitable
for use in our setting, though one of these infections was confirmed by microscopy to be \textit{falciparum} only. It is interesting to note that, we did not detect \textit{vivax} mono-infection. This species differentiation will help the clinician to determine the severity of malaria and specific treatment. Also, the laboratory will further be relief of the burden associated with preparation of thin film for the purpose of species differentiation.

The evaluation described in this study has demonstrated that SD Bioline RDT can be used as a screening test for malaria diagnosis in a health based facility with laboratory unit, especially during the time of high transmission rate of malaria. The use of the RDT in a setting like ours is useful to reduce the TAT of the malaria diagnosis. Indeed, laboratories in a resource constraint setting have to face the fact that reliable microscopy is not always available due to insufficient trained and experienced microscopists. In addition, the presence of only a single microscope that remains microscopy is not always available due to insufficient constraint setting have to face the fact that reliable microscopy is not always available due to insufficient trained and experienced microscopists. In addition, the presence of only a single microscope that remains microscopy is not always available due to insufficient

Overall, while the gold standard for malaria testing remains microscopy, but the limitations associated with this technique could affect the speed of delivery of quality services to the patients. Hence, the RDT can become the first screening test for the diagnosis of malaria in the laboratory. However, the cost of RDT analysis can be a burden to patients in a setting like ours, but this can be averted by interventions from the government and non-profit organizations considering its contribution to patient’s care.

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REFERENCES