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Histidine Rich Protein-II-based Rapid Diagnostic Test (HRP-2 RDT) assessment for malaria diagnosis in Bamenda urban settings in the North Western highlands of Cameroon

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Abstract

Background: Malaria is a long term Public Health Problem in tropical Countries including Cameroon. Prompt and accurate diagnosis is essential for an effective malaria control and elimination. Rapid Diagnosis Tests (RDTs) are widely used for malaria diagnosis with a continuous influx of various developed products in the market, identified by a wide variable field of performance.The objective of this study is to evaluate the performance characteristics of malaria Histidin Rich Protein-II-based on Rapid Diagnostic Test (HRP-2 RDT) in detecting malaria parasite for malaria diagnosis among different population groups in Western highlands of the North West region of Cameroon.

Materials and method: The cross-sectional study involved 60 febrile patients aged 6 months to 60 years and above, directed to the laboratory department at the Bamenda Regional Hospital for blood screening, after showing signs and symptoms of malaria infection, from the 30th of November to the 15th December 2016. Blood sample were collected aseptically, dispensed into EDTA coated tubes and investigated for the presence of malaria parasites using microscopy and the HRP-2 RDT.

Results: Of the 60 enrolled patients, 37 (61.7%) were found to be positive with blood film examination while 27 (45%) were positive with rapid diagnostic test. Based on frequency of infection by age, 20-35 years had 13 (35.1%) as the most vulnerable group, followed by 0-5 year with 11 (29.7%), 6-19 had 6 (16.2%) while age group of 36-59 had 5 (16.0%) and lastly the age group of 60 and above had least value of 2 (5.4%). The prevalence of malaria obtained through microscopy (62%) was significantly higher ($P<0.05$) than in RDT (45%). Considering microscopy as the gold standard, HRP-2 RDT exhibited high specificity (100%) but low sensitivity (73%) with positive predictive and negative predictive values of 100% and 70%, respectively. However, the sensitivity of HRP-2 RDT increased significantly with increase in *P. falciparum* parasitaemia which was the plasmodium species detected in all positive cases.

Conclusion: In this study, HRP-2 RDT revealed a reduced sensitivity as compared to routine microscopy test. The increased frequency of false-negative RDT results implies there is a need for designing and implementing alternative to RDT including next-generation, higher sensitivity diagnostic tools appropriate for these settings. However, while waiting for new techniques, RDT diagnosis should be followed by stained blood film microscopy in low transmission settings.

Keywords: Malaria, Microscopy, Sensitivity, specificity, and HRP-2 RDT.

Background

Malaria is a long-term deadly disease to mankind for over hundreds of years, in the humid tropical regions with warm temperatures particularly in Africa and South East Asia. The numbers of malaria infected cases are higher during the rainy season that results to development of more water pond which serve as breeding sites for malaria parasite and the abundance of green vegetation from agricultural activities. [1]. The World Health Organization (WHO) estimated that, 3.2 billion people are at risk of being infected with malaria and according to the latest estimates, 219 million cases of malaria occurred globally in 2017 and the disease led to 407,000 deaths [2]. Sub-Saharan Africa bears the highest burden of malaria, accounting for an estimated 90% of all malaria deaths worldwide of which more than 78% of malaria deaths occur in children aged <5 years. Over 90% of Cameroonians are at risk of malaria infection, and about 41% have at least one of malaria each year [3].

The main strategy for malaria control as recommended by the WHO includes a prompt and accurate diagnosis followed by effective treatment [4,5].The confirmation of suspected malaria cases by microscopy or a rapid

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diagnostic test (RDT) before treatment [5] will result in a significant reduction of morbidity and mortality related to misdiagnosis. Although PCR may represent the best diagnostic method with high sensitivity and specificity [6,7], it is still costly and not very useful for routine diagnosis [8]. Hence, microscopy and RDTs continue to be the primary choices for diagnosing malaria in most endemic countries. Microscopy is still considered the "gold standard" for malaria diagnosis in endemic countries because; it has a sensitivity of 50–500 parasites/µl [9], is far less expensive, and allows the identification of species and parasite density [10,11]. However the implementation of microscopy as diagnosis tool, especially in remote areas has been reported to be cumbersome and requires well trained personnel, high standard microscopes and a source of electricity [12].

In remote areas of sub-Saharan Africa, RDTs have become the primary tool for the parasitological diagnosis for the confirmation of malaria suspected cases. [13]. The most widely used RDTs for malaria are based on the detection of parasite histidine-rich protein II (HRP2) and *Plasmodium* lactate dehydrogenase (pLDH) [14]. The major constraints of RDTs are false positives, because HRP2 persists in the blood for several days after parasite clearance with antimalarial [15]. Another drawback of RDTs is the occurrence of, false-negative results reported to be associated to gene deletions [13]. This situation may delay the treatment process and increase the number of persons capable of infecting mosquitoes in the community. Moreover, RDTs are thought to be more specific to *P. falciparum* infections [16]. Variation of performance in the diagnosis method has been shown to have a great impact on individual treatment and epidemiological surveillance [17], and also some health professionals in endemic countries have lose confidence in test results and resolved to treat systematically all fever cases as malaria [12,18]. This may results to the over- or under-diagnosis of malaria, with excessive use of anti-malarial drugs or negligent treatment, which invariably contributes to malaria morbidity and the development of resistance [14,19]. Clinical diagnosis is imprecise but remains the basis of therapeutic care for the majority of febrile patients in malaria endemic areas, where laboratory support is often out of reach. Scientific quantification or interpretation of the effects of malaria misdiagnosis on the treatment decision, epidemiologic records, or clinical studies has not been adequately investigated [20]. Despite an obvious need for improvement, malaria diagnosis is the most neglected area of malaria research as laboratory supported diagnosis hardly reached majority of cases in remote areas living in endemic regions, and accounting for less than 0.25% ($700,000) of the U.S.$323 million investment in research and development in 2004 [20]. Rational therapy of malaria is essential to avoid non-target effects, to delay the advent of resistance, and to save cost on alternative drugs. Accurate diagnosis is the only way of effecting rational therapy. Confirmatory diagnosis before treatment initiation regained attention, partly influenced by the spread of drug resistance and thus the requirement of more expensive drugs that are unaffordable by low income countries [21]. Previous studies revealed that, quality RDT procurement does not necessarily guarantee an excellent field performance [22] and variable performance has been recorded, in terms of RDT sensitivity and specificity for malaria diagnosis in Cameroon [18,23,24,25] with lowest sensitivity reported during a low-transmission season [26]. If low performance rates are extrapolated, thousands of patients may be incorrectly diagnosed and receive inappropriate treatment resulting to low quality care and unnecessary drug use [27]. Furthermore, there is no standardized tool to be used by reference laboratories for a systematic monitoring of RDT performance and there is no post-marketing surveillance practice in most endemic countries [25]. Although the WHO provided specific programs for RDT quality evaluation, lot testing involved limited assessment of RDT buffer and accessories [20]. Besides, other factors including temperature, handling, transportation, quality of test kids and storage have been shown to affect the sensitivity of RDT test [22,28,29], thus the importance to continually explore the determinants of RDT sensitivity for an effective malaria diagnosis and treatment. The objective of this study was to assess the performance characteristics of Malaria PF HRP-2 based RDTamong different population age groups in hospital settings of the Bamenda urban city in North Western highlands of Cameroon.

Research Methodology

Study area

This study was carried out in the Bamenda highlands (17,300 km2 (6,680 square mi) located in the North western part of Cameroon between latitudes 5°40' and 7° North of the equator, and longitudes 9°45 and 11°10' East of the Greenwich Meridian [30]. The North-West region is a highland area with lowest altitude of 1050m on the plains of Bambui and highest altitude of 3011m, and average altitude of 2030.5m above sea level. The region is characterised by mountainous terrain and rugged topography with steep slopes and deep valleys. Temperature ranges between; 15^{o}c to 27^{o}C with mean annual temperatures of 21^{o}C [31]. Mean monthly rainfall varies across the region from 8mm in January in to 388mm in September, with a mean annual rainfall of 2400mm Dominant vegetation cover type is savannah grassland on the hill tops and mixed woodland forest at the valley of the slopes [30]. The Bamenda highlands experiences two main seasons of prolong eight months of wet rainy season from March till October and four months of dry season from November till February. The Bamenda highlands is a hypoendemic with seasonal malaria parasite transmission occurring at very low level

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and thehe main malaria vectors in the area are *An. gambiae*, *An. coluzzii* and *An. funestus* [32] with parasite inoculation rates varying from 4.9 to 11 infective bites/person/year in the highland areas of the North-West region [33].

Study design and participants

Malaria transmission in the Bamenda highlands is seasonal, with a high transmission between October to December and peak transmission between November to December. A cross-sectional study was conducted within Mezam division in North Western region of Cameroon from the 30th of November to the 15th December 2016 at the regional hospital in Bamenda. Out of a 136 suspected screen cases, a total of 60 patients consisting of 30 males and 30 females, with varying ages were enrolled and admitted based on RDT and to be confirmed by microscopy diagnosis after showing signs and symptoms of malaria infection without clinical sign and symptoms of severe malaria. Participants provided written informed consent before enrolment in this study. Blood samples were collected by venipuncture into test tubes containing EDTA. After mixing, the blood samples were used to determine the hematocrit and for the preparation of thick and thin slides for microscopy, and for the RDTs assay.

Microscopic analyses

When making thick film, a drop of blood was placed at the centre of the cream grease free slide and spread with the edge of another slide in a repeated coil shape to a diameter of approximately 2 cm. The slides were labelled and left horizontally to air-dry and well-kept to prevent them from dust and damage. They were then stained with 2ml of a 10% Giemsa solution and then washed after 10 minutess using clean water. As for the thin film, a drop of blood was placed at the centre of the cream grease free slides. The film were then spread using a smooth edged slide spreader. 2 ml of methanol were applied and allowed for fixation for a period of 2 minutes. After fixation, 2 ml of a 10% giemsa solution were applied on the fixed smear and then washed after 10 minutes using clean water. A drop of immersion oil were applied on dried stained slides (both thin and thick films) and examined microscopically for malaria parasites using 100X objective lens and results recorded [34]
The parasite density per microliter of blood is calculated as:

$$\frac{\text{Number of parasites X 8000}}{\text{Number of leukocytes}} = \text{parasites per microlitre}$$

RDT Analysis

The test device, buffer and specimen were allowed to equilibrate at room temperature (10ºC – 30ºC) prior to testing. The test cassette was removed from the foil pouch by tearing at the notch and then placed on level surface. Five microliters of whole blood were slowly added into the sample well. Then 3 drops of clearing buffer were added to the buffer well. A purple colour is observed as the sample moves across the result windows in the centre of the test device by capillarity. The results were read after 25 minutes. Only the control line appears for a negative test results while the presence of a control band and a unique HRP-2 line indicates an infection with *P. falciparum*. The absence of a control band signifies an invalid test and the tests were repeated.

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Data Analysis

Data was carefully entered in an Excel spread sheet and analysed using the statistical package for social sciences (SPSS) version 20 software. Proportions were calculated and the diagnostic performance was determined by calculating the test sensitivity, specificity, and predictive values.

Ethical consideration

The ethical approval for the study was provided by the Ethics Committees of the Ministry of Public Health Cameroon and was registered with controlled-trials.com at: https://clinicaltrials.gov/ct2/show/NCT02974348 under the registration number NCT02974348.

Results

The prevalence of positive test in both microscopy and RDT diagnosis was highest (33.4% and 35.1% respectively) among the 20 to 35 years age group (figure 1) and the least prevalence in both diagnosis was seen in 60 years and above age group (5.4% in microscopy and 3.7% in RDT).

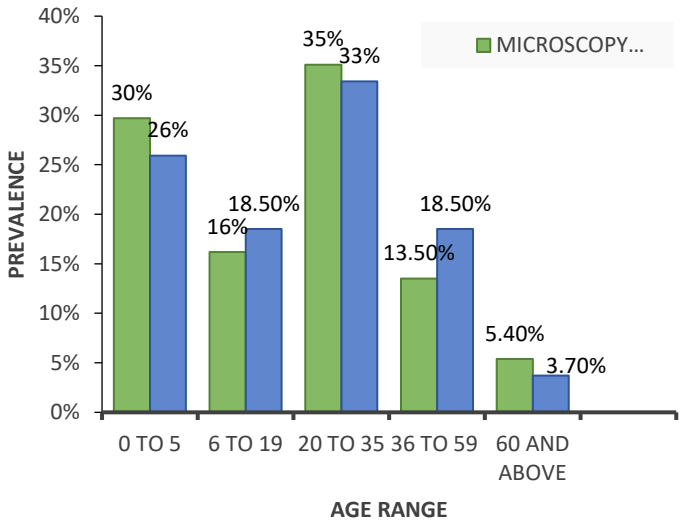

Figure 1: Distribution of diagnosis methods with respect to age group

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The overall prevalence of malaria parasites confirmed through microscopy (62%) was significantly higher ($P<0.05$) than in RDT (45%). Species of malaria parasite identified in all study participants was *Plasmodium falciparum*. The parasite density ranged from 40-400 per μL of blood. The number of subjects with true/false negative and true/false positive RDT's results are presented in table 1.

Table 1**:** No. of subjects with true/false negative and true/false positive RDT's diagnostic test results

TRUE FALSE	POSITVE	NEGATIVE	TOTAL
POSITIVE	**27**	**0**	**27**
NEGATIVE	**10**	**23**	**33**
TOTAL	**37**	**23**	**60**

Prevalence (RDT) =27/60=0.45=0.45×100=45%; Prevalence microscopy)=37/60=0.62=0.62×100=62%

This study revealed that RDT had a sensitivity of 73% indicating that RDT detected 73% of true ill patients, while 27% (false negative) could not be detected. Practically, this test detects 100% of malaria free patients. Implying that all patients diagnosed free of malaria parasites are actually healthy. PPV was 27/27 = 1 or 100% (table 2). Hence all patients who tested positive on RDT had the disease thereby indicating the presence of plasmodium falciparum parasite in the infected patients. Also among the 33 subjects diagnosed by RDT to be negative, 23 were true negative while 10 were false negatives giving a negative predictive value (NPV) of 23/33 = 0.70.This basically means that a person who test negative on RDT has a 70% likelihood of not having the disease.

Table 2**: Specificity, Sensitivity PPV and NPV of RDT and microscopy diagnosis**.

METHOD	SPECIFICITY	SENSITIVITY	PPV	NPV
RDT	**100**	**73%**	**100**	**70%**
MICROSCOPY	**100**	**100**	**100**	**100**

In this study routine microscopic examination of stained blood films which is considered as the gold standard for malaria diagnosis was able to detect more parasites than the RDT (sensitivity 73%). However, the specificity of 100% was similar to that of microscopy, distinguished by its high sensitivity and its ability to quantify parasitemia which is a good advantage.
The sensitivity of RDT is seen to be highest in age group of 20 to 35 years (53%) having a number of parasites ranging from 60 to 2720 (table 3), followed by 6 to 19 years (27%) with parasites ranging from 65 to 1200, 36 to 59 years (10.5%) with parasites ranging from 68 to 720, 0 to 5 years (6.6%) with parasites ranging from 128 to 2600 and least in age group of 60 years and above (2.9%) with parasites density ranging from 100 to 650.

Table 3: **RDT sensitivity with respect to number of parasites present in various age groups**.

Age group	Number of parasites per μl of blood	Sensitivity
0 to 5	128 – 2600	6.6%
6 to 19	65– 1200	27%
20 to 35	60 – 2720	53%
36 to 59	68 – 720	10.5%
60 and above	100 – 650	2.9%

Discussion

Microscopy and RDT, recommended by WHO, revealed to be the most reliable tools for prompt and accurate diagnosis prior to an effective treatment of malaria suspected cases[5]. The sensitivity of RDT reported in this study (73%) does not attain the 95% recommended by the World Health Organization [35]. This low sensitivity is disadvantageous as it will impair control intervention since a fraction of the infected population will be left untreated especially if RDT is the only available diagnostic tool. This could have important implications on health, transmission, and possibly morbidity and mortality. The sensitivity of RDT reported in this study is lower than previous reported in Nigeria [36], Zambia, Zanzibar and Thailand [34,37,38] but higher than the sensitivity recorded at the mount Cameroon areas [25] and in Gambia [39]. Importantly, this study recorded the specificity of 100% for RDT which is far higher than the one reported by previous studies in mount Cameroun and in Burkina Faso [28], but similar to the observation made among the Nigerian isolates [40].The difficulties and challenges faced by current RDTs have been reported showing a considerably varying specificities, sensitivities, numbers of false positives, numbers of false negatives and temperature tolerances of these tests [29]. False negative cases in this study were recorded for RDT using microscopy as diagnosis tool of reference. This false negative could be explained by the effect of PfHRP2 sequence variation on the binding of specific mAbs [13,41-44]. In contrast, this sequence variation of PfHRP2 DNA obtained from isolates from African and South America were not found to be linked to varying sensitivities of RDTs [45].This further reinforce the evidence that detection of malaria parasite by RDTs varies in different transmission settings, and emphasise the need for careful interpretation of prevalence estimates based on surveys employing RDTs alone [46]. Moreover, a prozone effect revealed to occur in PfHRP2 RDT [47]. and not with pLDH or aldolase based RDTs [48,49]. The storage temperature of RDT product by the marketers could also explain the observed low sensitivity. Other factors, including poor performance of specific RDT brands and lots, operator error, low-parasite density infections., storage and transportation conditions and training of health workers, handling methods may likely influence test performance [22,28,29,46,50].

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Interestingly, our study showed no false positive case and the absence of false positive test in this study is one of the a characteristic of a good test. This is interesting because accurate diagnosis is the basis for an adequate disease control and for delaying the emergence and spread of antimalarial drugs resistance [17]. Although, the rheumatoid factor cross-reacting in the blood has proven to generate a false positive test line, the issue is now resolved by developing new products in which IgG is being replaced by IgM [51]. Also, cross-reactivity with heterophile antibodies may be another cause of false positive test [52]. False positives have been recorded due to infection with *Schistosoma mekongi* [53]. False positives are also caused by the persistence of PfHRP2 in the blood for long periods after parasite clearance[9,54,55]. A reduced sensitivity of microscopy has been reported in the diagnosis of malaria infection in pregnant women due to placental sequestration of parasites. However, the detection of peripheral blood HRP-2 genes is possible with malaria parasites, making RDT very useful in such conditions [56]. But the diagnosis of placental infection by antigen detection without parasite density assessment by microscopy could have a significant impact on maternal and foetal health care. Besides, the difficulties associated with RDTs, such as genetic variability [7], including polymorphisms in the PfHRP2 gene highlights the importance for the development of alternative RDTs to the PfHRP2-based RDT including enzymes involve in parasite metabolic pathways such as the heat-shock protein 70, haem-detoxification protein and dihydrofolate reductase–thymidylate synthase [57,58]. Another promising alternative to RDTs is the PCR [6,7] , a molecular technique that has proven to be the most sensitive and specific method available as it allows the detection of parasitemia as low as 2–5 parasites/μl [3]. However, it is expensive, complex, and not appropriate for field use. Again, it has to be validated by the WHO before its implementation in health facilities.

In this study, it was noticed that a greater proportion of patients with positive RDT were diagnosed within the age ranges of 20 to 35. The high sensitive to RDT may be due to the large number of patients diagnosed within this age group as compared with 0 to 5; 6 to 19, 20 to 35 and 60 and above age groups respectively. Moreover, the optimal function of the immune system is optimal between the age ranges of 20 to 35 years [59,60], hence being able to detect the least amount of antigens in the system thus a greater sensitivity in RDT. Meanwhile infants (ranging from 0 to 5 years) who have a developing immune system are not highly sensitive to antigens in the system [61,62], hence a low sensitivity in RDT. The elderly (60 and above) have a suppressed immune system due to aging leads to less sensitivity of the test.

Conclusions

Stained blood film microscopy and rapid diagnostic test each with its characteristics, strengths and limitations together present the best hope for diagnosis as a key component of successful malaria control. Although RDT usefulness in low transmission areas or in the detection of low parasite density infections is being questioned, RDT it remains the only available alternative for malaria diagnosis in remote areas where microscopy diagnosis is absent and offers a good alternative, being an easy and rapid method that does not require an experienced laboratory technician. RDTs can act as a diagnostic tool to manage malaria in resource-poor settings with limited access to microscopy. Notwithstanding, the increased frequency of false negative RDT results in low transmission and urban settings implies that there is need for designing and implementing other fast and more sensitive alternative to RDT including next-generation, higher sensitivity diagnostics tools appropriate for malaria parasite in low transmission settings. However, while waiting for the implementation of these new techniques, PfHRP2-based RDT diagnosis should be followed by stained blood film microscopy to ascertain the degree of infection and to identify the malaria species involved for proper treatment in low transmission areas Besides, multidisciplinary research should continue to explore the determinants of RDT performance, and seek to better understand how to support and sustain the longevity of this diagnostic tool.

Competing interests

All authors declared no conflict of interest.

Authors' contributions

MTN and XNZ designed and supervised the experiments and data analysis. CTF performed sample collection and laboratory experiment. MTN, CTF and PFG drafted and wrote the manuscript. PFG reviewed and edited the manuscript. All authors read and approved the final manuscript.

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