

## Performance Evaluation of *Plasmodium falciparum* Histidine-Rich Protein 2 Rapid Diagnostic Test, when Compared to Microscopy

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### Abstract

**Introduction:** In malaria diagnosis, a highly sensitive and specific test will ensure appropriate administration of antimalarial treatment, hence promoting a parasite-based diagnosis as recommended by the World Health Organization (WHO). The Global Malaria Program recommends that suspected clinical malaria could be confirmed using the quality assured Rapid Diagnostic Test (RDT) and microscopy diagnostic tools. This study was designed to assess the performance of *Plasmodium falciparum* Histidine-Rich Protein 2 (PfHRP2-RDT), with respect to age and parasite density.

**Methodology:** This study was carried out in the Bamenda Regional Hospital Laboratory, with 381 patients enrolled into the study by convenient sampling technique. A simple questionnaire, microscopy and PfHRP2-RDT techniques were used to collect data on sex, age, and malaria status of the study participants. Both descriptive statistics and analysis of variance were used for data analysis.

**Results:** Results by microscopy show that up to 68.55% (109/159) of the males and 41.89% (93/222) of the females were infected. About 55.44% of those infected were younger children ( $\leq 5$  yrs) and young adults ( $>18$  yrs to  $\leq 35$  yrs), with up to 68.81% of the infections being mild parasitemia. Results by microscopy and PfHRP2-RDT were not the same, and the difference between the daily variation in test results was significant at  $P=0.0012$ . With microscopy as the standard, the sensitivity, specificity, positive predictive value and negative predictive value of PfHRP2-RDT were; 100%, 92.75%, 94.26% and 100% respectively.

**Conclusion:** The microscopy technique indicated low specificity and positive predictive values. Hence, in order to ensure an effective parasite-based malaria diagnosis, a microscopy confirmatory test is recommended for every PfHRP2-RDT positive result.

**Keywords:** *Plasmodium falciparum*; Microscopy; Histidine-rich protein; Rapid diagnostic test

### Introduction

The sensitivity of a test, which is its ability to accurately identify the presence of the infectious agent is as important as the specificity, which accurately identifies the absence of the infectious agents. In malaria diagnosis, a highly sensitive and specific test will ensure appropriate administration of antimalarial treatment, hence promoting a parasite-based diagnosis as recommended by WHO [1]. In malaria endemic settings, the rapid diagnostic test (RDT) and microscopy are suitable diagnostic methods for routine malaria clinical cases, which covers most of the microscopy and RDTs done in the public health sectors [2]. In fact, the Global Malaria Program recommends that suspected clinical malaria can be confirmed using the quality assured RDT and microscopy diagnostic tools [2]. That explains why malaria diagnostics with the largest impact on malaria control has been microscopy and RDTs [3]. However, these diagnostic techniques may be inappropriately used, due to inadequate laboratory support in malaria endemic areas where therapeutic management of febrile patients is frequently based on inaccurate clinical diagnosis [3]. Nonetheless, with proper quality control and quality assurance system, the microscopy method can be accurately used in diagnosing malaria as the cause of febrile illness.

However, marked inadequacy in the quality control system may, amid other factors contribute to the recurrent impaired malaria diagnosis by the microscopy method reported even in hospital-based laboratories [4,5]. Therefore, there is need for a more convenient and less complicated procedure in malaria diagnosis. The malaria RDT is the current alternative which fits that need. Although RDT sensitivity reduces with reduced level of malaria parasitaemia ( $<500/\mu\text{L}$  for *P. falciparum*), according to WHO, it should reach at least 95% in order

to be a helpful diagnostic tool [6]. In order to conveniently rely on RDT as a necessary substitute for the microscopy technique, this study was designed to evaluate the performance of PfHRP2-RDT, in the Bamenda Regional Hospital Laboratory within the periods of April to June 2018. Specifically, this study was designed to assess the performance of PfHRP2-RDT, using microscopy as the standard.

### Background literature

Factors like poor techniques in slide preparation, heavy work load, poor condition of the microscope, poor quality of laboratory supplies and insufficiently handled skilled microscopy will cause poor malaria diagnosis [7]. But with proper quality control and assurance system in place, microscopy can be used to quantify and identify malaria parasite species. In fact, it was reported that asexual parasites can be detected by a skilled handling of the microscope at a density of  $<10$  parasites per  $\mu\text{L}$  of blood [8]. However, the sensitivity reduces to  $<100$  parasites per  $\mu\text{L}$  in field conditions [8]. Alternatively, the RDT procedure is less complicated, with generally cost-beneficial kits requiring very little

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to be effectively run. Although a few factors such as environmental conditions in the manufacturing process, may affect RDT performance [9,10]. RDTs generally require little operator training. Nonetheless, malaria parasites cannot be quantified and parasite species identified with RDT, it however prevents missed diagnosis of malaria or febrile illnesses with different etiologies [7].

Studies which considered microscopy as the gold standard found that RDT exhibited low sensitivity and high specificity [11,12]. In a malaria endemic zone, when compared to film microscopy the sensitivity, specificity, PPV and NPV of RDT were 82.2%, 100.0%, 100.0% and 34.3%, respectively, with a significant difference between both test methods [13]. Meanwhile in a hypo endemic zone, the sensitivity, specificity, PPV and NPV of RDT were 90.0%, 99.9%, 90.0% and 99.9%, respectively [14]. And in a meso endemic zone, the sensitivity, specificity, PPV and NPV of RDT were 91.0%, 65.0%, 71.6% and 88.1% respectively [14]. Studies have even shown that false positive RDT results are associated to high rheumatoid factor levels, leishmaniasis, hepatitis C, Schistosomiasis, toxoplasmosis, human African trypanosomiasis, dengue and Chagas disease [15,16]. Individuals with history of malaria and children were also found to be associated with false positive RDT results [17]. Due to low-density infection, sensitivity and PPV were low, in Swaziland, a low-transmission area [18]. A statistically significant association was found between malaria positivity rate and male, children below five years of age and those with fever more than 24 hours before diagnosis [18]. Although the sensitivity and positive predictive values of RDT were low, higher values were reported in patients with fever, as compared to non-febrile patients [18]. Consequently, the specificity of RDT and even its cost-effectiveness can be affected not only by the presence of some infections, but also by age, malaria endemicity, season and the presence of fever [14].

Malaria antigen target RDTs are immunochromatographic assays which uses monoclonal antibodies on a test strip, to detect malaria antigen in a small amount of blood. Histidine-Rich Protein 2 (HRP-2) which is specific to *P. falciparum* is the most frequent malaria antigen target in RDTs. Although HRP-2, has been shown to remain in the blood of the patient for weeks even after successful treatment, *Plasmodium falciparum* HRP-2-RDT is still considered a good laboratory test for malaria detection at low-level, in chronic cases [19]. It has even been reported that the sensitivity of HRP-2 tests was frequently greater than 90% [8,20]. RDTs are generally considered an effective diagnostic tool of malaria, which are easy to perform [21]. The highly sensitive and stable RDTs that detect the histidine-rich protein 2 (HRP2) antigen is recommended in endemic areas where *P. falciparum* is dominant [22]. PfHRP2-RDT is also recommended even with the availability of RDTs which detects the enzyme parasite lactate dehydrogenase (pLDH), produced by all four human *Plasmodium* species [22]. But due to the persistence of HRP2 for several weeks after treatment, HRP2-based tests have been reported to show high number of false positives, resulting in low specificity [23,24]. Furthermore, the HRP2 protein has been reported to show variation in its repeat section which may be the cause for extensive variation in the sensitivity of HRP2-based RDTs [25]. According to another findings, RDT will be a useful substitute where there is high parasite density [13,14]. In fact, the HRP2-based RDT was shown to have higher sensitivity, as compared to microscopy in malaria diagnosis [26].

## Materials and Methods

### Ethical consideration

The ethical clearance for this study was gotten from the Ethical

Review Committee of the University of Bamenda. Written informed consent were gotten from those who accepted to be enrolled into the study, while informed assent was taken from the parents/guardians of minors.

### Study area and population

This study was carried out in the Bamenda Regional Hospital (BRH), the principal government hospital in the North West Region of Cameroon. Bamenda which is one of the ten regional headquarters in Cameroon is located 5.96 latitude and 10.15 longitude and situated at the height of 1258 meters above the sea level. There is both the dry and the rainy seasons in Bamenda, with a balance rainfall per year being 2064 mm (and 172 mm per month). The peak of dry season occurs in January, meanwhile the peak of rainy season is in September. The BRH is part of the Bamenda Health District (BHD), which is made up of many public, private and mission health facilities, located within the 17 health areas in the BHD. The BRH therefore functions as the referral hospital in the region, with an estimated 337,036 inhabitants [27].

### Sample collection

Approximately 2-3 mL of venous blood samples were collected into EDTA anticoagulated test tubes, from a total of 381 patients who were sent to the laboratory for a malaria test. Blood films (thick and thin) were prepared within a period of 30 minutes, following the techniques recommended by Cheesbrough et al., and the manufacturer's instruction (Carestart TM Malaria HRP2 (Pf), produced by ACCESS BIO, INC. 65 Clyde Rd. Suite A, Somerset, NJ 08873, USA) [28].

**RDT method:** Following the manufacturer's instruction, the malaria RDT Carestart TM Malaria HRP2 (Pf) test strips were used to test each sample. The Carestart TM Malaria HRP2 (Pf) is used for the diagnosis of *P. falciparum* infection.

**Microscopy test method:** The prepared blood films were processed and stained with 3% Giemsa staining technique [28]. Two experienced microscopists who were unaware of the RDT results independently examined duplicate slides. A third experienced microscopist confirmed results with discrepancies. Parasite density per microlitre of blood was estimated following the methods in a previous study [14].

### Statistical analysis

Both descriptive and inferential statistics were used to analyze the primary data generated from laboratory analysis of the blood samples. Analysis of variance was used to access the daily effect of using RDT, in relation to the standard microscopy test method.

## Results

Table 1 shows the distribution of study participants according to sex. A total of 381 patients were examined, out of which 222 (58.27%) were females while 159 (41.73%) were males. Results by microscopy show that up to 68.55% (109/159) of the males and 41.89% (93/222) of the females were infected. But by RDT, 71.08% (113/159) of the males and 46.40% (103/222) of the females were infected. Therefore, 56.69% and 53.02% of the study participants were infected according to RDT and microscopy respectively.

|                                          | Females     | Males       | Total       |
|------------------------------------------|-------------|-------------|-------------|
| <b>Number Examined</b>                   | 222 (58.27) | 159 (41.73) | 381         |
| <b>Number Infected by RDT (%)</b>        | 103 (46.40) | 113 (71.07) | 216 (56.69) |
| <b>Number Infected by Microscopy (%)</b> | 93 (41.89)  | 109 (68.55) | 202 (53.02) |

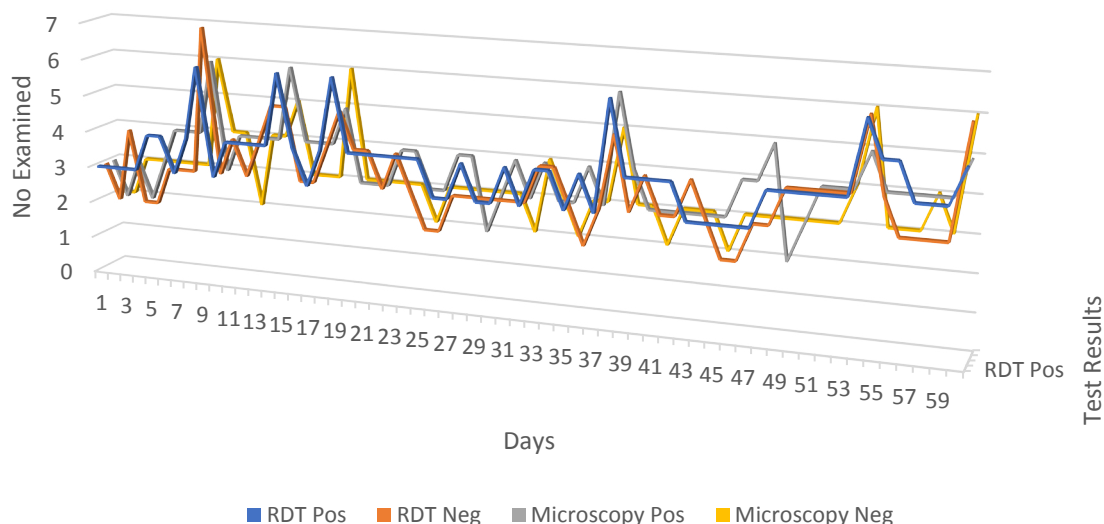
**Table 1:** Distribution of participants according to sex and test results.

The results presented in Figure 1, justifies the links between those tested positive and negative, based on the microscopy and RDT techniques. It can be observed from the different values that, the findings from both techniques are not the same, hence suggesting the presence of differences between the two methods. However, is there any significant difference between the two methods? Since descriptive statistics has no critical value, the study was therefore advanced by using the analysis of variance (ANOVA) to test whether significant difference exists between the two methods.

The quantitative results as presented in Table 2a reveals that, the analysis of variance F-statistic value is 5.453 with degree of freedom 3:236 and probability value of 0.0012. By implication, the calculated value of F-statistics is greater than its table value of 2.144 and it is statistically significant at 1% level of significance. The study therefore observes that there is significant difference between the daily variation in test results produced by RDT and microscopy.

Table 2b shows the sensitivity, specificity, positive and negative predictive values of the RDT methods, using the microscopy test method as standard. While the sensitivity and Negative predictive values were all 100%, the specificity and positive predictive values were 92.75% and 94.26% respectively.

Table 3 shows the degree of malaria parasitaemia and RDT results, against ages of the patients. Out of the 202 patients who tested positive by microscopy, approximately 27.72% were young children, 09.90% children, 27.72% young adults, 17.82% middle aged adults and 16.83% older adults. In terms of parasite density, out of the 202 positive cases, 68.81% were mild parasitaemia (100 to 500 parasites/ $\mu$ L of blood), 03.47% Mild Parasitaemia (>500 to <1000 parasites/ $\mu$ L of blood), 14.85% moderate parasitaemia ( $\geq$  1000<10000 parasites/ $\mu$ L of blood) and 12.87% severe parasitaemia ( $\geq$  10000 parasites/ $\mu$ L of blood). Considering microscopy as standard, there were 14 false positive results.



**Figure 1:** Daily distribution of microscopy and RDT test results (Source: Generated by authors using Eview 7).

| Sample: 381 - Included observations: 60 days |       |          |                    |                        |
|----------------------------------------------|-------|----------|--------------------|------------------------|
| Method                                       |       | df       | Value              | Probability            |
| Anova F-statistic                            |       | (3,236)  | 5.452821           | 0.0012                 |
| Analysis of Variance                         |       |          |                    |                        |
| Source of Variation                          |       | df       | Sum of Sq.         | Mean Sq.               |
| Between                                      |       | 3        | 14.31250           | 4.770833               |
| Within                                       |       | 236      | 206.4833           | 0.874929               |
| Total                                        |       | 239      | 220.7958           | 0.923832               |
| Category Statistics                          |       |          |                    |                        |
| Variable                                     | Count | Mean     | Standard Deviation | Standard Error of Mean |
| RDTP                                         | 60    | 3.900000 | 0.837672           | 0.108143               |
| RDTN                                         | 60    | 3.450000 | 1.032112           | 0.133245               |
| MICP                                         | 60    | 3.683333 | 0.892372           | 0.115205               |
| MICH                                         | 60    | 3.250000 | 0.967699           | 0.124929               |
| All                                          | 240   | 3.570833 | 0.961162           | 0.062043               |
| Note: Generated by authors using Eview 7     |       |          |                    |                        |

**Note:** Generated by authors using Eview 7

**Table 2a:** The Summary of Analysis of Variance Results.

| Sensitivity (%) | Specificity (%) | Positive Predictive value (%) | Negative Predictive Value (%) |
|-----------------|-----------------|-------------------------------|-------------------------------|
| 100             | 92.75           | 94.26                         | 100                           |

**Table 2b:** Sensitivity, specificity, positive and negative predictive values of RDT using microscopy as gold standard.

|                                      | Young children | Children           | Young Adults        | Middle aged Adults  | Older Adults | Total (%)   |
|--------------------------------------|----------------|--------------------|---------------------|---------------------|--------------|-------------|
|                                      | ≤ 5yrs         | >5 yrs to ≤ 18 yrs | >18 yrs to ≤ 35 yrs | >35 yrs to ≤ 55 yrs | >55 yrs      |             |
| Total Examined                       | 89             | 36                 | 100                 | 76                  | 80           | 381         |
| Mild Parasitaemia (100 to 500)       | 36             | 14                 | 34                  | 28                  | 27           | 139 (68.81) |
| Mild Parasitaemia (>500 to <1000)    | 0              | 0                  | 0                   | 4                   | 3            | 7 (03.47)   |
| Moderate Parasitaemia (≥ 1000<10000) | 10             | 6                  | 6                   | 4                   | 4            | 30 (14.85)  |
| Severe Parasitaemia (≥ 10000)        | 10             | 0                  | 16                  | 0                   | 0            | 26 (12.87)  |
| Total Positive by Microscopy (%)     | 56 (27.72)     | 20 (09.90)         | 56 (27.72)          | 36 (17.82)          | 34 (16.83)   | 202         |
| Total Positive by RDT (%)            | 63 (29.17)     | 23 (10.65)         | 56 (25.93)          | 37 (17.13)          | 37(17.13)    | 216         |

Units for parasitaemia is parasites/μL of blood

**Table 3:** Degree of malaria parasitaemia and RDT results according to ages of the patients.

## Discussion

Although more females (58.27%) than males (41.73%) were enrolled into the study, more of the males were rather found to be malaria positive. According to the microscopy results, 68.55% (109/159) of the males and 41.89% (93/222) of the females were infected. That shows a male sex bias in malaria prevalence amongst the patients examined in this study. Although females are generally considered more susceptible to malaria, the males might have also been more exposed to the infection through outdoor activities at peak mosquito biting periods. With Bamenda being a cosmopolitan city, it is expected to inhabit males from all works of life. The males might not have also been actively involved in practices like use of mosquito bed nets and other preventive measures, hence exposing themselves to the malaria. Additionally, other studies have shown that men involved in outdoor activities during mosquito biting peak period, and women involving in household chores which exposes them to mosquito bites are at greater risks to contracting malaria [29,30]. Therefore, to think that malaria is gender blind simply because mosquitos do not discriminate in their biting habit, may be wrong. Some factors could possibly encourage gender-biased acquisition of malaria. In line with a retrospective study, there was a report on an adult male bias in *P. falciparum* and *P. vivax* infections, in a hypo endemic area for malaria [31]. Conversely, in another study mortality from severe falciparum malaria was found to be significantly higher in the non-pregnant females than males [32]. Although there may be higher malaria mortality in females, most studies rather report higher malaria prevalence in males, when pregnant women are not considered [18,33].

As observed from Figure 1, the numbers of positive and negative results produced by both microscopy and RDT are not the same. Furthermore, the analysis of variance results on Table 2 shows significant difference ( $P=0.0012$ ) between the daily variations in the test results produced by RDT and microscopy. With microscopy as the standard, out of the 216 positive results by RDT, 14 were false positives. A possible explanation for the presence of false positive results is the persistence of HRP2 protein in the blood stream, even after treatment [23,34]. Although history on malaria illness was absent, the possibility of previous treatment for *P. falciparum* malaria, probably resulting to the persistence of HRP2-protein in their blood stream cannot be rejected. The possible presence of unknown infections may have also interfered with the sensitivity of the HRP2-RDT results, producing the observed variation in the HRP2-RDT and microscopy methods [13,14]. Although no significant difference was found between microscopy and the HRP2-RDT methods in some selected health facilities in the cape coast metropolis of Ghana, due to incomplete correlation, authors did not recommend RDT. This study however did not report parasite burden, which has been reported to affect RDT sensitivity [13,14,26,35]. As rightly recommended by another author, more research in different

malaria transmission settings and clinical situations is needed to determine the level of agreement between microscopy and RDT [36].

Compared to microscopy, the sensitivity, specificity, PPV and NPV of the HRP2-based RDT for this study were 100%, 92.75%, 94.26% and 100% respectively. This means good sensitivity and NPV, but low specificity and PPV. It further means that, the HRP2-RDT used in this study accurately identified the presence of malaria, but not its absence. However, WHO recommends both high sensitivity and specificity for an appropriate parasite-based diagnosis [1]. Contrary to our findings, many studies reported low sensitivity for the HRP2-RDT test [11,12,18]. In fact, the specificity and negative predictive values of RDT were good, while the sensitivity and positive predictive values were reportedly low, but higher in patients with fever [18]. However in another study where microscopy was compared to HRP2-RDT, HRP2-RDT was even reported to show superior sensitivity [26]. High HRP2-RDT sensitivity with increase in parasite density was also reported [26,35]. Despite the low parasite density in this study, where up to 68.81% (139/202) of those infected had mild parasitaemia, the RDT sensitivity was still good. These discrepancies may have been due to the differences in malaria endemicity.

This study was done in a high malaria endemic zone. Another study which compared PfHRP2 based RDT and microscopy reported a relatively low sensitivity and specificity (68.7% and 80.4% respectively) for the RDT method in a mesoendemic malaria transmission zone [12]. In areas of low malaria transmission, it was recommended that, new malaria diagnostic tools with high sensitivity was required, because both the HRP-2 malaria RDT and microscopy showed sensitivity which was less than PCR [37]. PCR and RDT were earlier found to be poor in identifying mixed *Plasmodium* spp infections [38]. Since it was also suggested that the low sensitivity in the HRP2-RDT may be caused by extensive variation in the HRP2 protein repeat region there might have been a lack of such variation among the *P. falciparum* species in our study population [25].

In this study, most of those infected were young children ( $\leq 5$  yrs) and young adults ( $>18$  yrs to  $\leq 35$  yrs). In fact, 27.72% of those infected were young children and 27.72% were young adults. Incidentally, patients with severe parasitaemia were only found among these age groups. However, the least percentage of those infected were children ( $>5$  yrs to  $\leq 18$  yrs). The high malaria prevalence among children below 5 years can be attributed to their low level of acquired immunity against malaria. In fact, below five years of age was found to be statistically associated to positive malaria test. High malaria prevalence in young adults who are of the active age group ( $>18$  yrs to  $\leq 35$  yrs) suggests that, their daily activities probably exposes them to bites by infected mosquitos. Ten out of the fourteen patients with false positive results were young children. This further indicates a possible association

between false RDT results and the younger age group, equally reported in another study [17]. It has been suggested that RDT be given preference to microscopy only when the patient's age is over 13 years [12]. Although in suspected malaria cases within the ages 5 years and older, the mean operational sensitivity of RDTs in nine health facilities in Tanzania was low (64.8%), there was great variation among the health facility (range 18.8-85.9%) [35]. Consequently, age appears to have an influence on the RDT performance. It was further suggested that RDT specificity also appears to be generally affected by age [14]. Therefore, in order to appropriately determine RDT performance, the influence of age should not be ignored.

## Conclusion

Based on the microscopy technique, there was a high malaria prevalence rate of 53.02% (202/381) among the study population, with 68.81% (129/202) of the infected cases being mild parasitemia. The difference between the daily results by microscopy and PfHRP2-RDT was statistically significant at  $P=0.0012$ . In the absence of mixed infections, the PfHRP2-RDT method has shown good sensitivity (100%), but relatively poor specificity (92.75%) with microscopy as the standard. Considering the good sensitivity, PfHRP2-RDT appears to be a suitable substitute for microscopy. However, judging from the negative predictive value (100%) and the positive predictive value (94.26%), a negative result proved reliable, but not a positive one. Also because of the low specificity (92.75%), a microscopy confirmatory test is recommended for every PfHRP2-RDT positive result. This study was however limited in that, the presence of other infections which could have affected the sensitivity/specificity of the PfHRP2-RDT was not tested. Additionally, the history on malaria infections in the patients was absent. For further studies, study participants should be screened for all possible current infections which could hinder the PfHRP2-RDT test specificity.

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